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Serum-Free, Long-Term Cultures of Human Hepatocytes: Maintenance of Cell Morphology, Transcription Factors, and Liver-Specific Functions

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Since human hepatocytes are available only in limited number, the development of a serum-free culture system for long-term cultivation of differentiated and functional hepatocytes is of great importance. Here we describe the culture of human hepatocytes in a chemically defined serum-free medium for up to 5 weeks. Cell morphology was assayed by light and electron microscopy and revealed a well-preserved cellular morphology. Marker proteins for epithelial and bile duct cells, cytokeratin (CK) 18 and 19, and liver-specific proteins, like phosphoenolpyruvate carboxykinase-2 (PCK2) and serum proteins, were expressed. Liver-enriched transcription factors CCAAT/enhancer binding protein α (C/EBPα) and hepatocyte nuclear factor-4 (HNF-4), cytokine and mitogen activated factors (nuclear factor kappa B) NFκB, and activator protein-1 (AP-1) were maintained and active for several weeks in our cultures. In summary, our serum-free culture system allows the culture of differentiated human hepatocytes for several weeks. It may serve as a model system for metabolic, pharmacologic–toxicologic studies, and studies on human pathogens under defined chemical conditions.

Key Words: **human hepatocytes; cell morphology; cytokeratins; transcription factors; phosphoenolpyruvate carboxykinase; serum proteins.**

The liver, as central organ of energy metabolism, biotransformation of xenobiotics, and synthesis of plasma proteins under normal conditions and injury, maintains many vital processes in mammals. Primary cultures of hepatocytes have been and still are an important system to study liver-specific functions. So far, metabolic studies have primarily been performed in rat hepatocyte cultures. However, interspecies differences have been reported with regard to apolipoprotein (apo) A-IV expression (1), cytochrome P450 induction (2), metabolic regulation of cholesterol and triacylglycerol synthesis (3), and the intracellular distribution of gluconeogenic enzymes (4–7). Therefore, the long-term cultivation of primary human hepatocytes seems to represent a more appropriate experimental system for the evaluation of liver specific processes in man, the biology of human viral pathogens or parasites, and drug metabolism in phase I and II reactions (8, 9).

The major obstacle in establishing culture conditions that allow these investigations is the maintenance of liver specific functions. Several different attempts have been made using different substrates (10), culture media and hormonal conditions usually in the initial or ongoing presence of serum (11, 12). So far, most studies were done in short-term cultures, namely the cells were maintained for maximally 8 days.

Long-term maintenance of static human hepatocyte cultures for several weeks has been described in a few reports either in the presence of serum (13), or in a system that involves the transfer of cells from a conventional two-dimensional to a three-dimensional sandwich system (14). While especially in the first study a number of liver specific functions including cytochrome P450 expression were assayed, the second study relied on only two donors and was restricted to data on serum protein expression. In a completely different approach, with the primary goal set on achieving a high growth potential, a long-term culture of hepatocytes was established in the presence of a vari-

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ety of different sera and conditioned media (15). Maintenance of liver-specific functions is usually monitored by the expression of serum proteins, which is regulated by liver-enriched transcription factors like HNF-4 (16–18) and by transcription factors that activate or repress genes in response to injury and inflammation, like AP-1 or NFκB. On the other hand, a number of transcription factors interfere with HNF-4 by competing for identical DNA-binding sites, thereby repressing the expression of liver-specific genes (19, 20). However, there is only limited knowledge on the maintenance and activity of transcription factors in long-term human hepatocyte cultures (21).

Recently, we have established culture conditions that allow serum-free cultivation of human hepatocytes in the presence of Hepatocyte Growth Factor (HGF) and Epidermal Growth Factor (EGF). Here we show that hepatocyte morphology, hepatocyte specific gene expression and functions are maintained for at least 4–5 weeks. We monitored the level and activity of several transcription factors, protein kinases, and serum protein expression. This culture system may serve as an appropriate model system to analyze regulation of liver specific processes in man.

MATERIALS AND METHODS

The Δ5 variant of human HGF was a gift from Snow Brand Milk Products (Tochigi, Japan), natural mouse EGF was purchased from Collaborative Research (Bedford, MA). Vitrogen (bovine collagen type 1) was from Collagen Corp. (Fremont, CA). Gentamycin, Penicillin/Streptomycin, DMEM with and without Glucose (DMEM #11965 and #11966), and MEM (#41600) was purchased from (GIBCO/BRL, Gaithersburgh, MD). PCK2 antisera was generated in rabbit against a peptid corresponding to amino acids 558–571 (22). All other primary antibodies were obtained from Santa Cruz Biotechnologie Inc. (Santa Cruz, CA). Secondary antibodies were obtained from Sigma (St. Louis, MO). Culture dishes were from Corning Costar (Cambridge, MA). RNAzol B was from Tel-Test (Friendswood, TX), X-omat film was purchased from Kodak (Rochester, NY), GeneScreen Membranes from NEN (Boston, MA). Albumin mRNA was detected with an 0.9 kb Pst I fragment derived from plasmid pHA68B12, transferrin mRNA was detected using a 0.8 kb Pst I fragment from plasmid TF66G2 both obtained from the ATCC (Rockville, MD, ATCC #59713 and #57228). Mitochondrial phosphoenolpyruvate carboxykinase (PCK2) mRNA was detected using a 1.4 kb mtPCK2 cDNA fragment (22), for the detection of β-actin mRNA a 500 bp ss-actin cDNA fragment was used as hybridization probe. ApoA-I, apoA-IV, apoC-III, glycerolaldehydphosphate dehydrogenase (GAPDH), apolipoprotein regulator protein-1 (ARP-1), erb-A-related (EAR)-2, and EAR-3 (=COUP-TF1 chicken ovalbumin upstream promotor transcription factor1) were detected as described previously (23, 24).

Isolation and culture of human hepatocytes. Human hepatocytes were isolated out of excess liver tissue from reduced human liver transplant procedures or from donor livers that had not been used for transplantation (for donor history see Table 1). Hepatocytes were isolated by perfusion with collagenase-P (Boehringer Mannheim, Indianapolis, IN) through the existing vasculature, as described previously (25). Cells were plated onto collagen coated plates and cultured as described recently (21). Briefly, the initial plating was done in MEM containing 500 ng/ml insulin and 50 μg/ml gentamycin for 3–12 h. The medium was then changed to a mixture of DMEM (no glucose):DMEM (plus glucose):MEM (25:25:50), with the following additions 1 g/l albumin, 5.5 mM galactose, 0.5 mM glutamine, 0.3 mM ornithine, 0.13 mM proline, 5 mM Hepes, 2.5 ng/ml Na-selenite, 2.5 μg/ml transferrin, 0.2 mM $ZnCl_2$, 0.04 mM $CuSO_4 \times 5\ H_2O$, 0.13 mM $ZnSO_4 \times 7\ H_2O$, 0.1 mM $MnSO_4$, 2.75 μg/ml insulin, 25 μg/ml gentamycin, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 nM dexamethasone, 40 ng/ml HGF, and 20 ng/ml EGF. To simplify, we will refer to this medium composition as Human Hepatocyte Maintenance Medium (HHMM) with HGF and EGF.

TABLE 1

Donor History

Donor	Gender	Age [years]	Cause of death	Cell viability [%]
622	F	4	Car accident	89
638	M	0	2-day-old with gastroschisis	98
642	F	32	Boating accident	82
660	M	2	Drowned	85
673	M	44	Fall from scaffold on concrete	79
701	M	3	Cardiopulmonary arrest	87

Note. The cell viability was assessed by the trypan blue exclusion tests. M, male; F, female.

Protein isolation and Western blot analyses of whole cell lysates. Native protein extracts were isolated and subjected to Western blot analyses as described (21). For determining protein concentrations Bicinchonic acid Protein Assay (Sigma, St. Louis, MO) was used. SDS polyacrylamide gel electrophoresis was performed according to Laemmli (26).

Nuclear protein analysis. Nuclear proteins were isolated, protein, Western blot analyses, and electrophoretic mobility shift assays were performed as described earlier (27).

RNA isolation and Northern blot analyses. RNA was isolated and subjected to Northern blot analyses for albumin and transferrin as described (27). PCK2 and β-actin mRNA were detected as follows: the membrane with the transferred electrophoresed RNA was preincubated in 5 ml of hybridization solution containing 5× SSC, 5× Denhardt's solution (1× Denhardt's is 0.02% Ficoll 400/0.02% polyvinylpyrrolidone/0.2% BSA), 0.5% SDS and 500 μg of salmon sperm DNA at 68°C for 30 min in a hybridization tube with continuous rolling in a Biometra hybidization box (0V 3). Each cDNA probe (15 ng) was labeled by the random-priming method using [α-^{32}P]dCTP as the labeled nucleotide and the Klenow fragment of DNA polymerase (Megaprime, Amersham). The radioactively labeled DNA probes, each at 10^6 cpm/μl, were denatured for 5 min at 95°C, then chilled quickly on ice and added to the hybridization solution. Blots were hybridized at 68°C for 12 h, rinsed three times in 2× SSC/0.05× SDS, then washed once for 40 min at room temperature and twice for 20 min in 0.1× SSC/0.1× SDS at 50°C. They were analyzed overnight using a PhosphorImager (Molecular Dynamics, Krefeld, Germany). The data were scanned by IMAGEQUANT software from Molecular Dynamics.

Transmission electron microscopy. Cells grown on collagen-coated tissue culture plates for various times were fixed in 2.5% glutaraldehyde in PBS (8 gm/l NaCl, 0.2 gm/l KCl, 1.15 gm/l $Na_2HPO_4 \times 7H_2O$, 0.2 gm/l KH_2PO_4, pH 7.4) overnight at 4°C. Monolayers were washed in PBS three times then post-fixed in aqueous 1% osmium tetroxide, 1% Fe_6CN_3 for 1 h. Cells were washed 3 times in PBS, then dehydrated through a 30–100% ethanol series, then several changes of Polybed 812 embedding resin (Poly-

sciences, Warrington, PA). BEEM capsules filled with resin were placed on top of the infiltrated monolayers then the entire tissue culture plate was cured overnight at 37°C, then cured for two days at 65°C. BEEM capsules containing the hepatocyte monolayers were pulled from the plate then sectioned enface at 60 nm using a Riechart Ultracut E microtome, post-stained in 4% uranyl acetate for 10 min and 1% lead citrate for 7 min. Sections were viewed on a JEOL JEM 1210 electron microscope at 80 KV.

RESULTS

Cell morphology. Cell morphology was assayed in serum-free cultures on collagen coated plates by light and electron microscopy (Fig. 1). The cells showed a typical morphology of mature hepatocytes for up to 31 days, mainly mono- and binuclear cells were present (Fig. 1, upper panel, A–C). Round shaped nuclei, mitochondria and Golgi apparatus were well established and maintained from early days in the culture until at least day 24. At all time points, bile canaliculi (C) were readily apparent. Glycogen (G), and desmosomes (D) also developed around day 11, indicating metabolic activity as well as close cell-cell contacts. The apparent increase in glycogen over time is probably caused by the prolonged incubation of the hepatocytes in the presence of insulin and glucose. (Fig. 1, lower panel, A–D). It is evidend that although hepatocytes are by far the most prominent cell type, the cultures consist of a mixed population of cells (Fig. 1, lower panel, B). CK18 and CK19, serving as marker proteins for epithelial and bile duct cells were expressed throughout the culture for up to 48 days, a representative experiment in shown in Fig. 2. PCK2, the mitochondrial isoform of the gluconeogenic key enzyme PCK1, is encoded in the nucleus. PCK2 mRNA (not show) and protein were present throughout the culture independent of the donor (Fig. 2), thereby demonstrating the functionality of protein synthesis.

Expression of transcription factors. Previous reports have suggested that the isolation procedure with disruption of cell-cell and cell-matrix contacts exerts major stress on hepatocytes (28). In human hepatocytes a new steady state level for STAT(signal transducer and activator of transcription)-3 and for liver enriched factors, C/EBPα, HNF-3, HNF-4, was established by day 2 and maintained for several days (21). All proteins were expressed throughout the whole culture period at the same level. Only slight changes in DNA binding activities were observed until day 15 (21). Therefore, we started to investigate transcription factor levels from day 4 on.

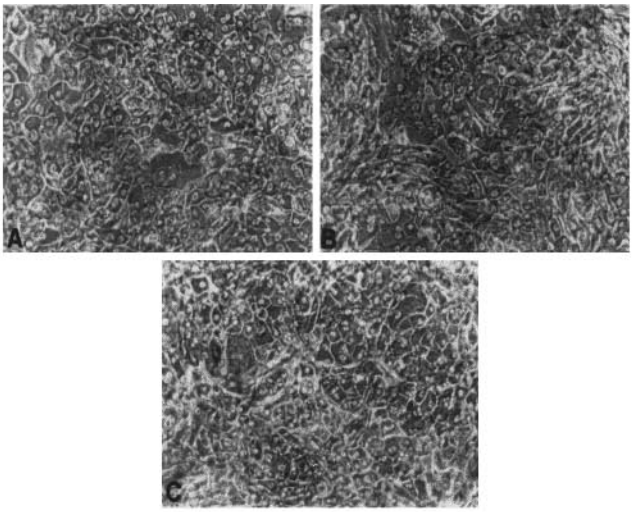
Since the binding site used to monitor C/EBP-DNA binding activity in our previous study (21) is recognized by all the different C/EBP isoforms, we performed Western blot analyses to determine the levels of C/EBPα in nuclear extracts. C/EBPα was expressed at a constant level until day 21 (Fig. 3), and is therefore at least in part responsible for the observed DNA binding activity. HNF-4 protein was expressed with minor variation in whole cell extracts, one representative experiment is shown in Fig. 3. In addition, HNF-4-DNA-binding activity was detectable for up to 36 day in culture (not shown). Furthermore, we monitored the expression of ARP-1, EAR-2, and EAR-3 by Northern blot analyses. While ARP-1 mRNA levels were very low at early time points in the cultures, mRNA amounts increased at later time points (Fig. 4). Also, ARP-1 protein levels seemed to increase during prolonged culture (Fig. 3). EAR-3 mRNA was not detectable within the first 2–3 weeks of culture. A prominent increase was observed at day 21 (donor 622) and around day 28 and 36 (donor 660). EAR-2 mRNA levels were barely detectable, but they remained fairly constant throughout the culture period. The variations, as seen in Fig. 4, correlated well with the variations observed for GAPDH mRNA which was used as an internal control.

NFκB expression was monitored by Western blot analyses and electrophoretic moblitiy shift assays. In whole cell lysates NFκB p50 and p65 subunits were present throughout the culture period independent of the donor, a representative experiment is shown in Fig. 3. NFκB DNA binding activity was also maintained in the cultures (Fig. 5), even in the absence of potent inducers like interleukin-6.

Mitogens like HGF or EGF can indirectly activate protein kinases like MAP kinases and JNKs (Jun N-terminal kinases) that in turn phosphorylate c-Jun and c-Fos which enables them to form the AP-1 complex and bind to DNA to activate or repress specific genes. Components of this signal transduction pathway like MAP-kinase and JNK were expressed in our cultures (data not shown). c-Fos and c-Jun proteins were expressed throughout the culture period and electrophoretic mobility shift assays revealed that AP-1 DNA binding activity was detectable in the hepatocytes for up to 36 days (Fig. 5).

Expression of liver-specific proteins. We used the expression of serum proteins as markers of liver-specific function. Albumin mRNA was detected by Northern blot analyses in 3 different cultures. Albumin

FIG. 1. Morphology of human hepatocytes in long-term serum-free cultures. Upper panel: Phase contrast of human hepatocytes maintained in culture over 4 weeks. Hepatocytes were cultivated in HHMM with 40 ng/ml HGF and 20 ng/ml EGF for (A) 11 days, (B) 17 days, and (C) 31 days. Lower panel: Transmission electron microscopy of human hepatocytes (donor 622) maintained in culture over 3–4 weeks. Cells were maintained in culture for (A) 4 days, (B) 8 days, (C) 11 days, and (D) 24 days. At all time points, bile canaliculae (C) are readily apparent. As culture matures, glycogen (G), and desmosomes (D) also develop around day 8, indicating metabolic activity as well as close cell–cell contact of the hepatocytes. Bar, 2 μm.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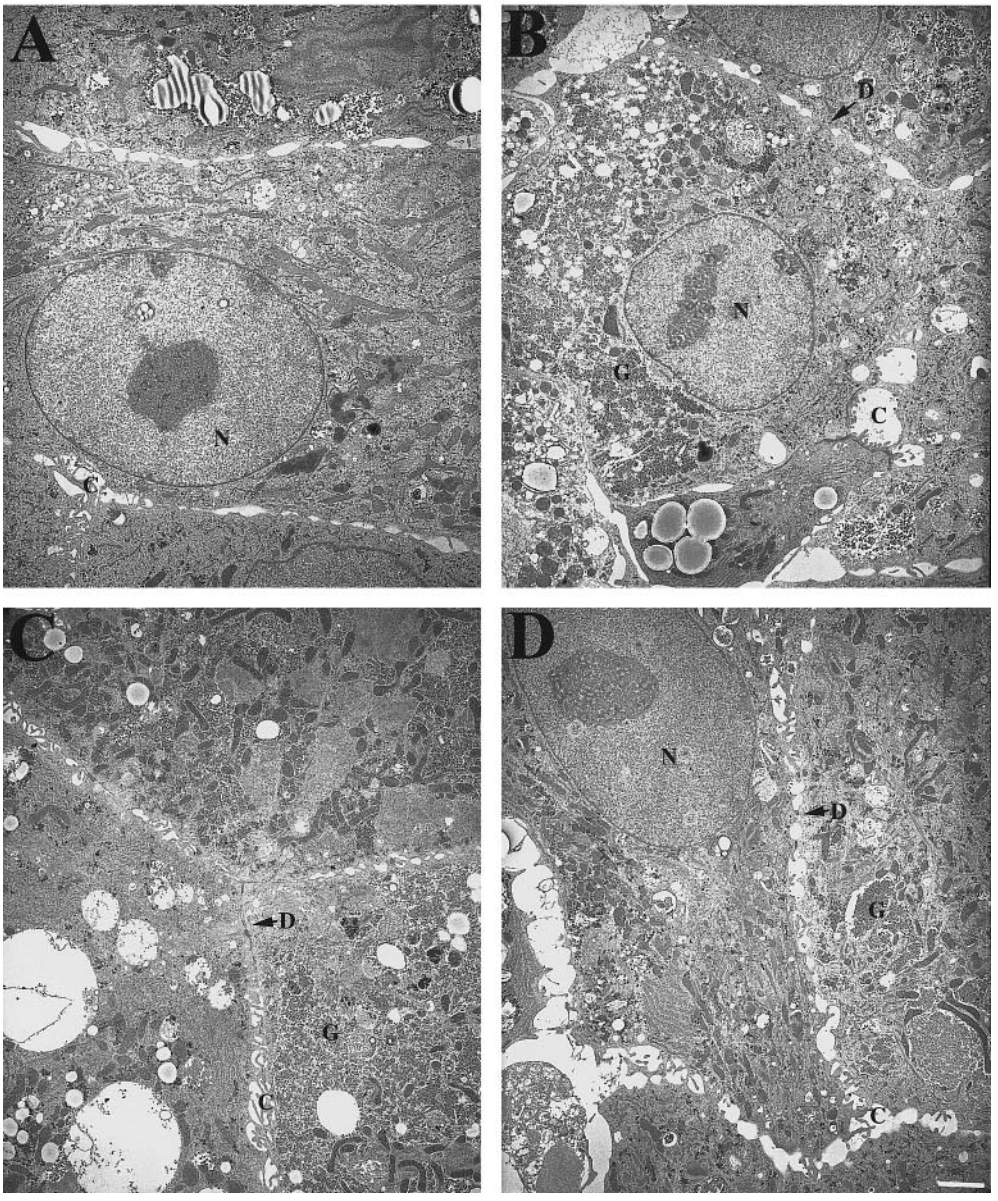
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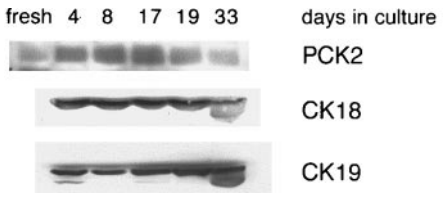

FIG. 2. Expression of cytokeratins and phosphoenolpyruvate carboxykinase-2 in human hepatocyte cultures. Human hepatocytes were cultured in HHMM with HGF and EGF. At times indicated, whole cell lysates were prepared and subjected to SDS–PAGE and Western blot analysis. Filters were probed with specific antisera as indicated. Representative results were obtained from donor 622.

mRNA was expressed at high levels in freshly isolated cells. In 2 cultures (donors 622 and 660) albumin mRNA was expressed at lower levels than in freshly isolated hepatocytes. However, albumin mRNA expression was maintained throughout the whole culture period with only minor variations. In two cultures (donors 622 and 673) albumin mRNA amounts increased during prolonged culture, reaching about 3 fold higher levels on days 28 and 36 as compared to day 7 (Fig. 6). Even though transferrin was exogenously added to HHMM, transferrin mRNA was detectable throughout

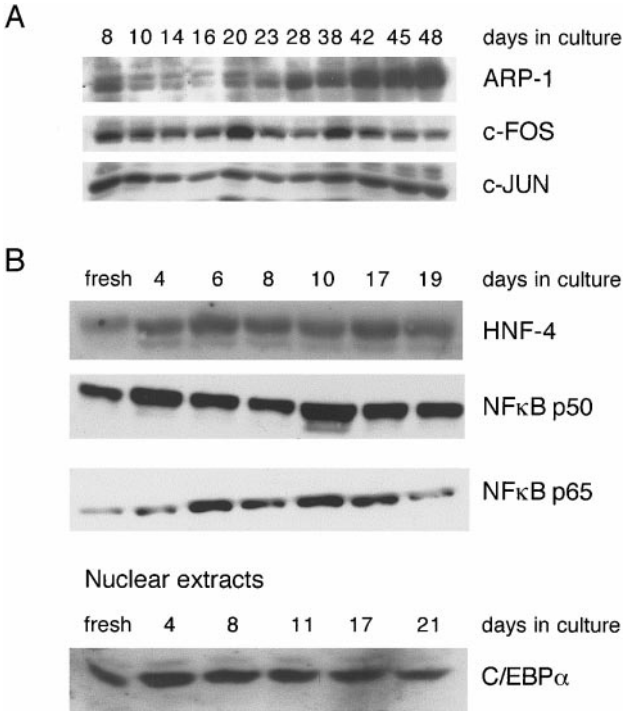

FIG. 3. Expression of transcription factors in long-term serum-free human hepatocyte cultures. Hepatocytes were cultured in HHMM with HGF and EGF until days indicated. 30 μg of nuclear protein extract (C/EBPα) or 50 μg of whole cell extracts were separated by SDS–PAGE and transferred onto Immobilon membranes. The membranes were probed with a specific antisera as indicated. Representative results are shown for C/EBPα, HNF-4, NFκB p50, and NFκB p65 (donor 622), c-Fos, c-Jun, and ARP-1 (donor 660).

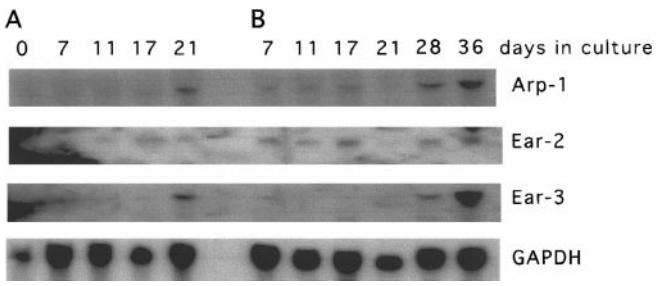

FIG. 4. Expression of ARP-1, EAR-2, and EAR-3/COUP-TF1 mRNAs in long-term serum-free human hepatocyte cultures. Hepatocytes were cultured in HHMM with HGF and EGF. The cells were harvested at indicated times, total RNA was isolated, and Northern Blot analyses were performed. Filters were hybridized with specific cDNA probes as indicated and normalized using a probe for GAPDH. Representative results are shown for ARP-1, EAR-2, and EAR-3/COUP-TF1 (A, donor 622; and B, donor 660). The membranes were probed with a cDNA for GAPDH for normalization.

the culture period (Fig. 6). Next, we investigated the expression of proteins involved in lipid and cholesterol transport. ApoA-I, and C-III mRNAs were present throughout the culture period, at every time point investigated (Fig. 6). As a negative control we investigated the expression of apoA-IV, which is expressed in humans in intestine, but not in liver (1). We were unable to detect expression of apoA-IV (Fig. 6).

DISCUSSION

Primary human hepatocytes were cultured serum-free for at least 48 days. The cells are characterized by a well preserved morphology and function including the storage of glycogen, expression of plasma proteins for at least 36 days (Figs. 1 and 6). Cell-cell contacts were established and maintained throughout the culture period. Bile canaliculi and cellular organelles like mitochondria were preserved as shown by electron microscopy and the expression of marker proteins CK19 and PCK2, the mitochondrial isoform of phosphoenolpyruvate carboxykinase (Figs. 1 and 2).

Expression of Transcription Factors

(A) Liver enriched factors C/EBP and HNF-4. Establishing and maintening the liver-specific functions

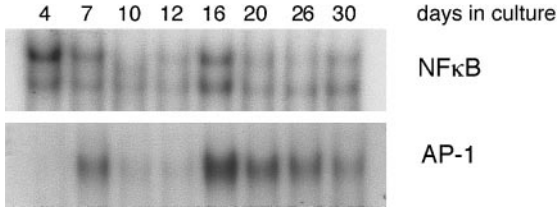

FIG. 5. AP-1 and NFκB DNA binding activity in human hepatocyte cultures. Hepatocytes (donor 701) were cultured in HHMM with HGF and EGF. At day indicated nuclear extracts were prepared. Electrophoretic mobility shift assays were performed using specific DNA binding sites for AP-1 and NFκB.

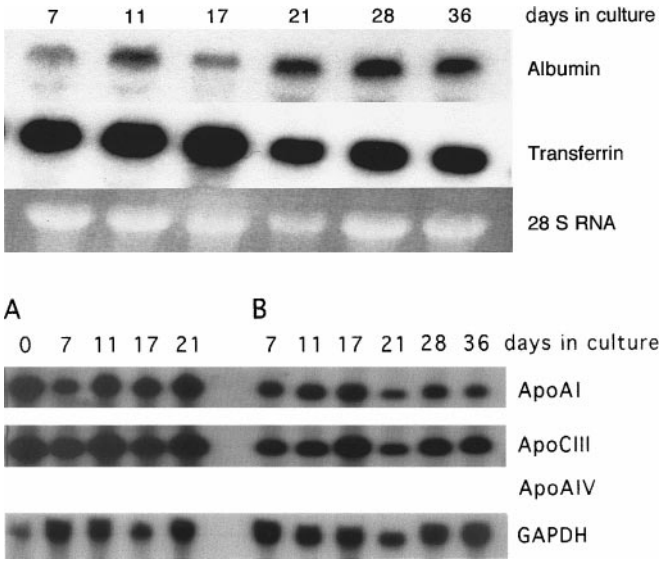

FIG. 6. Expression of serum proteins in long-term serum-free human hepatocyte cultures. Hepatocytes were cultured in HHMM with HGF and EGF. The cells were harvested at indicated times, total RNA was isolated, and Northern blot analyses were performed. Filters were hybridized with specific cDNA probes as indicated and normalized using a probe for 28S RNA or GAPDH. Upper panel: representative blots are shown for albumin and transferrin (donor 673). Lower panel: representative blots are shown for apolipoprotein A-I, A-IV, and C-III (donors 622 and 660). Apolipoprotein A-IV mRNA was not detectable at any given time point, the row is left blank intentionally.

and thereby the differentiated state of the hepatocytes is thought to be regulated by a coordinated interplay of hepatocyte-specific transcription factors including C/EBPα and HNF-4 (29). In primary cultures of rat hepatocytes C/EBPα-protein expression is rapidly reduced within a few days of culture, but can be maintained on a constant level by the addition of matrix proteins (Matrigel) onto the cultures. This expression pattern is reflected by the differentiation status of the cells, where liver-specific proteins are expressed when C/EBPα levels are high (27). HNF-4 is involved in hepatocyte specific expression of serum proteins and of cytochrome P450 proteins (16, 30, 31). HNF-4 is an activator of HNF-1 which in turn activates a large number of genes involved in carbohydrate and fatty acid metabolism (32, 33). The maintenance of HNF4 and C/EBPα for at least 3 weeks in culture (Fig. 2) is most likely the reason for the prolonged expression of liver specific proteins albumin, transferrin, apoA-I and apoC-III (Fig. 6).

(B) Expression of ARP-1, EAR-2, and EAR-3/COUP-TF1. ARP-1, and EAR-3/COUP-TF1 are transcription factors known to compete with HNF-4 for specific DNA-binding sites in the promotor regions of transferrin and apolipoprotein genes. ARP-1, EAR-3/COUP-TF1 mRNAs were expressed at low levels during the first 3–4 weeks of hepatocyte culture, and were increased only at later stages from day 21 on (Fig. 4). Also ARP-1 protein levels seemed to increase with ongoing culture (Fig. 3). EAR-3/COUP-TF1 and their homologues are also known to play a role in determining the cell fate during developmental processes (34). Therefore, the increase in mRNA- and protein-levels of ARP-1 and of EAR-3/COUP-TF1 might indicate the beginning of a transition to a more de-differentiated state of the hepatocytes.

(C) Expression of cytokine or growth factor inducible transcription factors. NFκB, a protein associated with hepatocyte proliferation and prevention of apoptosis after partial hepatectomy (35–37), is expressed in proliferating rat hepatocyte cultures (38). Although the hepatocytes in our cultures proliferate only to a low degree (21), we were able to detect p50 and p65 subunits of NFκB, both able to bind DNA for at least 30 days (Figs. 3 and 5). NFκB is known to be activated by TNFα, which is increased in serum in states of acute liver failure. Since we also detected STAT-3 (21, 39) this suggests that activation of NFκB is mediated by STAT-3 in this culture system.

The heteromeric transcription factor AP-1 consists of Jun and Fos proteins and is activated in response to a great number of stimuli, including mitogenic growth factors and inflammatory cytokines (40). c-Jun, c-Fos and basal AP-1 DNA binding activity were present in nuclear extracts for up 48 days in our cultures (Figs. 3 and 5). Activation of AP-1 by growth factors occurs via members of the mitogen activated protein kinase family like MAPK, ERK (extracellular signal regulated kinase), and JNK/SAPK (c-Jun N-terminal kinase/stress activated protein kinase). JNK1, JNK2/3, and MAPK p42 and p44 were continuously expressed in our cultures (not shown). Therefore, they are most likely involved in maintaining AP-1 DNA-binding activity in hepatocyte culture in the presence of EGF and HGF. In summary, this system seems to be suitable to study cytokine as well as growth factor mediated events in human hepatocytes under defined chemical conditions.

Expression of Liver Specific Proteins

Serum proteins. Albumin is expressed in the liver and responsible for establishing serum colloid osmotic pressure and transporting fatty acids in the blood. Its expression is transcriptionally regulated (41). Transferrin is secreted by hepatocytes and functions as a transport protein for iron and as a growth factor for a variety of cells. Its expression is transcriptionally regulated in part by HNF-4 and C/EBP (16). Albumin and transferrin mRNA were expressed in our cultures, showing that mechanisms responsible for liver specific gene transcription are maintained. The genes for apoA-I, A-IV and C-III are tandemly organized in a multi-gene cluster localized on the long arm of human chromosome 11 (42). ApoA-I is the major protein com-

ponent in high density lipoproteins (HDL). HDLs are thought to play an important role in artherosclerosis prevention and longevity. Plasma HDL levels are correlated with plasma apoA-I protein and in turn with liver apoA-I mRNA levels (43). In rat hepatocyte cultures apoA-1 expression is lost within a few hours. ApoA-I expression is regulated by HNF-3, HNF-4, retinoid X receptor (RXR)-α, and ARP-1, transcription factors that are interacting with a hepatocyte specific enhancer in the promoter region of the apoA-I gene (17, 18) and by estrogen receptors via the recruitment of the auxiliary factor RIP 140 (44). ApoC-III is present in the plasma in apoB containing lipoprotein and in non-apoB-containing lipoprotein. It inhibits the lipolysis of triglyceride-rich particles and the binding of apoB-containing lipoprotein to the low density lipoprotein receptor (45). ApoA-I and apoC-III mRNAs were expressed, independent of the donor and throughout the culture period (Fig. 6). This shows again the functionality of liver-specific gene transcription mechanisms in our cultures. We were unable to detect apoA-IV mRNA, which is in agreement with its expression pattern in rat liver, but not in human liver (1).

In summary, we demonstrate that the culture system presented here is a useful tool to study human hepatocyte specific gene expression in health and disease and the importance of relying on a human system in research. It may serve as model system for the study of human specific viral and parasite infections, drug metabolism and may allow culturing of hepatocytes for transplantation purposes, in cases where the introduction of therapeutically genes followed by autologous liver cell transplantation is desirable.

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