

Liver Endothelial Cells Promote LDL-R Expression and the Uptake of HCV-Like Particles in Primary Rat and Human Hepatocytes

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Low-density lipoprotein (LDL) is an important carrier of plasma cholesterol and triglycerides whose concentration is regulated by the liver parenchymal cells. Abnormal LDL regulation is thought to cause atherosclerosis, while viral binding to LDL has been suggested to facilitate hepatitis C infection. Primary hepatocytes quickly lose the ability to clear LDL during *in vitro* culture. Here we show that the coculture of hepatocytes with liver sinusoidal endothelial cells (LSEC) significantly increases the ability of hepatocytes to uptake LDL *in vitro*. LDL uptake does not increase when hepatocytes are cocultured with other cell types such as fibroblasts or umbilical vein endothelial cells. We find that LSECs induce the hepatic expression of the LDL receptor and the epidermal growth factor receptor. In addition, while hepatocytes in single culture did not take up hepatitis C virus (HCV)-like particles, the hepatocytes cocultured with LSECs showed a high level of HCV-like particle uptake. We suggest that coculture with LSECs induces the emergence of a sinusoidal surface in primary hepatocytes conducive to the uptake of HCV-like particles. **In conclusion**, our findings describe a novel model of polarized hepatocytes *in vitro* that can be used for the study of LDL metabolism and hepatitis C infection. *Supplementary material for this article can be found on the HEPATOLOGY website (<http://interscience.wiley.com/jpages/0270-9139/suppmat/index.html>). (HEPATOLOGY 2006;43:257-265.)*

Low-density lipoprotein (LDL) is a plasma-carried particle whose lipid component includes cholesterol and triglycerides.¹ LDL originates from very low-density lipoprotein (vLDL) synthesized by the liver with apoprotein B-100. The vLDL is converted to LDL by an endothelial lipase, which releases free fatty acids, increasing the density of the particle to form LDL. Excess

LDL is then taken up by hepatocytes through LDL receptor (LDL-R)-mediated endocytosis.¹

Improper hepatic clearance of LDL results in elevated plasma levels of LDL which is a serious risk factor for the development of atherosclerosis.² One of the first steps in atherosclerosis is the passage of LDL into the vascular wall. Once trapped in the vessel wall, LDL can undergo oxidation.³ Oxidized LDL is no longer a ligand for the LDL receptor.⁴ Accumulation of oxidized LDL and LDL aggregates in the vessel wall stimulates an inflammatory response causing endothelial injury, macrophage recruitment, foam cell formation, and smooth muscle cell proliferation.³ Current therapeutic intervention includes administration of statins, which block cholesterol production and increase expression of the LDL-R by the liver parenchymal cells,² removing LDL from circulation.⁵ In addition, liver sinusoidal endothelial cells (LSECs) remove oxidized LDL from the circulation via the scavenger cell receptor.⁵

LDL- and LDL-R-mediated pathways have also been shown to play a role in hepatitis C virus (HCV) infection.⁶ The HCV surface receptors, glycoproteins E1 and E2, have been shown to associate with LDL and vLDL.⁷ The virus particle itself was shown to associate with the

Abbreviations: LDL, low-density lipoprotein; LSEC, liver sinusoidal endothelial cell; HCV, hepatitis C virus; vLDL, very low-density lipoprotein; LDL-R, low-density lipoprotein receptor; PBS, phosphate-buffered saline; acLDL, acetylated LDL; DiI-LDL, 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate-labeled LDL; TNF- α tumor necrosis factor α ; HGF, hepatocyte growth factor; EGF, epidermal growth factor; EGF-R, epidermal growth factor receptor.

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LDL-R expressed on HepG2 cells.^{6,8} These results suggest that HCV might use the LDL-R to enter the cell and escape degradation.⁹ However, studies of LDL- and LDL-R-mediated interactions in primary hepatocytes have been limited, because the LDL-R is either nonexpressed or minimally expressed in primary hepatocyte cultures.

Current state-of-the-art culture methods for primary hepatocytes include: (1) culture in a collagen double-gel configuration,^{10–12} (2) coculture with fibroblasts,^{13–15} and (3) spheroid formation on Matrigel.^{16,17} Analysis of the hepatic synthetic function (albumin, urea) and of hepatic metabolism (cytochrome P450 activity) showed that these three methods can maintain a high level of liver-specific function for over 4 weeks *in vitro*. However, analysis of LDL uptake in cultured hepatocytes was limited to nonpolarized cell lines such as HepG2, possibly due to loss of LDL-R expression in primary cultured hepatocytes.^{6,18,19}

Here we demonstrate that primary rat and human hepatocytes show little uptake of LDL in a collagen double-gel cultures or when cocultured with fibroblasts. However, hepatocytes show a high level of LDL uptake when cocultured with LSECs on a single layer of collagen, or when cultured in spheroid form on Matrigel. Furthermore, we show that coculture with LSECs induces LDL-R expression in hepatocytes and the ability of hepatocytes to take up HCV-like particles. These results suggest that LSECs induce the emergence of a sinusoidal surface in primary hepatocytes, which is conducive to the uptake of HCV-like particles.

Materials and Methods

Reagents and Antibodies. Fetal bovine serum, phosphate-buffered saline (PBS), Dulbecco's modified Eagle medium, penicillin, streptomycin, trypsin-EDTA, and FITC-labeled acetylated LDL (acLDL) were obtained from Invitrogen Life Technologies (Carlsbad, CA). 1,1'-Diiododecyl-3,3',3'-tetramethyl-indocarbocyanine perchlorate-labeled LDL (DiI-LDL), and radiolabeled [¹²⁵I] LDL were purchased from Biomedical Technologies (Stoughton, MA). Growth factor-reduced Matrigel and fibronectin were from BD Biosciences (San Jose, CA). Hydrocortisone was obtained from Pharmacia (Kalamazoo, MI). Glucagon and Insulin were purchased from Eli-Lilly (Indianapolis, IN). Endothelial growth medium was obtained from Cambrex Corp. (East Rutherford, NJ). Recombinant rat tumor necrosis factor α (TNF- α) and hepatocyte growth factor (HGF) were obtained from R&D Systems Inc. (Minneapolis, MN). Immunofluorescence grade paraformaldehyde was purchased from Electron Microscope Sciences (Hatfield,

PA). Unless otherwise noted, all other chemicals were purchased from Sigma-Aldrich Chemicals (St. Louis, MO). For immunofluorescence studies, normal donkey serum and secondary F(ab')₂ antibody fragments, ML grade, were obtained from Jackson ImmunoResearch (Bar Harbor, ME). Sheep anti-EGF receptor 1:10 was purchased from Fitzgerald (Concord, MA). Neutralizing antibodies against rat TNF- α , HGF, and LDL-R were purchased from R&D Systems Inc. (Minneapolis, MN).

Hepatocyte Isolation and Culture. Rat hepatocytes were harvested from adult female Lewis rats (Charles River Laboratories, Boston, MA) weighing 150–200 g by way of a two-step *in situ* collagenase perfusion technique modified by Dunn et al.¹¹ Hepatocyte viability after the harvest was greater than 90% based on trypan blue exclusion. All animals were treated in accordance with National Research Council guidelines, and the study was approved by the Subcommittee on Research Animal Care at Massachusetts General Hospital. Hepatocyte purity was greater than 95%. Human hepatocytes (#5200) were purchased from ScienCell Research Laboratories (San Diego, CA) and cultured according to the manufacturer's instructions.

Hepatocytes were seeded directly on 6-well, collagen gel-coated plates at a density of 100,000 cells/cm². Cocultures were created by adding a second cell type (endothelial cells or 3T3-J2 cells) at 100,000 cells/cm² 4 hours after the addition of hepatocytes. Culture medium consisted of Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated fetal bovine serum, 200 U/mL penicillin/streptomycin, 7.5 μ g/mL hydrocortisone, 20 ng/mL EGF, 14 ng/mL glucagon, and 0.5 U/mL insulin. Hepatocyte cultures and cocultures were maintained in a 10% CO₂-humidified incubator at 37°C.

Nonparenchymal Cell Isolation and Culture. Rat LSECs were isolated from the nonparenchymal section of the liver using a two-step Percoll gradient (25%/50%) separation, following the procedure of Zhang et al.²⁰ Cells were counted following differential adhesion using a Beckman Coulter Z2 Particle Count and Size Analyzer (Fullerton, CA). LSEC purity was greater than 94% as assessed by DiI-acLDL uptake, HGF staining, and lack of stellate cell autofluorescence. Human LSECs (#5000) were purchased from ScienCell Research Laboratories (San Diego, CA) and cultured according to the manufacturer's instructions.

Human umbilical vein endothelial cells were purchased from Cambrex Corp. and cultured according to the manufacturer's instructions. Endothelial cells were used at passage <5. Cells were maintained in a 5% CO₂-humidified incubator at 37°C.

3T3-J2 fibroblasts were purchased from the American Type Culture Collection and cultured in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated fetal bovine serum, 200 U/mL penicillin/streptomycin. Cells were cultured in standard T-75 flasks and split 1 to 3 when 80% confluent. Cells were maintained in a 5% CO₂-humidified incubator at 37°C.

LDL Uptake. DiI-LDL was added to serum-free culture medium at 20 µg/mL concentration. Cells were incubated with the DiI-LDL solution for 3 hours, unless otherwise noted, in a 10% CO₂-humidified incubator at 37°C. For the modified LDL uptake studies, FITC-conjugated acLDL was added to the DiI-LDL solution at a concentration of 20 µg/mL. Cells were thoroughly washed with PBS to remove unbound LDL and were imaged in Phenol red-free Dulbecco's modified Eagle medium. To compare DiI-LDL uptake, random images were taken under identical camera gain and exposure settings, and average intensity was calculated using image-processing software. Uptake was normalized to hepatocyte-LSEC cocultures from the same isolation cultured under identical conditions. The quantification DiI-LDL uptake was verified using radiolabeled LDL [¹²⁵I]. Briefly, cells were incubated as previously described with 20 µg/mL [¹²⁵I] LDL for 3 hours. Excess radiolabeled LDL was washed, and the cells were trypsinized off the surface. Cell suspensions were subsequently analyzed using a Perkin-Elmer (Packard) Cobra Auto Gamma Counter (Boston, MA). Results confirmed the validity of the fluorescence assay.

Immunofluorescence Microscopy. Plates were washed 3 times with PBS and fixed in 4% EM-grade paraformaldehyde for 10 minutes at room temperature. Slides were then washed with PBS and incubated in 100 mmol/L glycine for 15 minutes to saturate reactive groups. Samples were permeabilized for 15 minutes with 0.1% Triton X-100, blocked for 30 minutes with 1% bovine serum albumin and 5% donkey serum at room temperature, and stained with primary antibodies overnight at 4°C. After additional washes with PBS, samples were stained with fluorescently tagged secondary antibodies for 45 minutes at room temperature.

TNF-α Detection via ELISA. LSECs were seeded on fibronectin-coated tissue culture dishes at a 100,000 cells/cm² density in culture medium supplemented with vascular endothelial growth factor (10 ng/mL). TNF-α was detected in the supernatant after 48 hours of culture using the Quantikine ELISA kit (R&D Systems, Inc.) according to manufacturer's directions.

Total RNA Isolation and Reverse-Transcriptase Polymerase Chain Reaction. RNA was extracted from 4 × 10⁵ rat hepatocytes cultured with or without LSECs

on collagen. Messenger RNA was analyzed via one-step reverse-transcriptase polymerase chain reaction (1 cycle at 55°C for 30 min and 95°C for 15 min, then 27 cycles at 94°C for 60 s, 62°C for 30 s, and 72°C for 60 s) using 1.5 ng of total RNA as a template and specific primers for rat LDL-R and rat EGF receptor (EGF-R). Primer sequences are available by request.

Expression and Purification of HCV Envelope Heterodimers E1 and E2. Plasmid (pShuttle/E1_{FLAG}E2)²¹ encoding the HCV E1 and E2 proteins—in which highly variable region 1 was exchanged with a FLAG epitope—was a generous gift from Shoshana Levy. Properly folded HCV envelope E1 and E2 heterodimers were expressed and purified as described by Cocquerel et al.²¹ Briefly, COS-7 cells (American Type Culture Collection) were transiently transfected using the Mammalian Transfection Kit (Stratagene, La Jolla, CA) with pShuttle/E1_{FLAG}E2, then washed and lysed in 1% Triton X-100. Clarified cell lysates were preadsorbed onto a protein A agarose column (Sigma) and then applied to a calcium-dependent anti-FLAG M1 affinity column (Sigma). Purified HCV heterodimers were dialyzed against PBS overnight at 4°C and concentrated. Purified proteins were analyzed via silver staining.

Binding of E1, E2 Heterodimers to Fluorescent Beads. Green-fluorescent carboxylated beads 100 nm in diameter were purchased from Polysciences, Inc. (Warrington, PA). HCV envelope E1 and E2 heterodimers were covalently linked to the beads using a Carbodiimide Kit with Hollow Fiber Filtering System (Polysciences, Inc.) according to the manufacturer's instructions.

Results

LDL Uptake in Hepatocyte Culture and Coculture.

LDL clearance from the blood is an important function of the liver parenchymal cells.²² To study hepatic uptake of LDL *in vitro*, we seeded rat hepatocytes either alone or in coculture with 3T3-J2, human umbilical vein endothelial cells, or LSECs on collagen gel (Fig. 1A). Twenty-four hours after seeding, the cultures were washed with PBS and incubated with serum-free medium containing 20 µg/mL DiI-LDL for 3 additional hours. The cells were subsequently washed to remove nonspecifically bound DiI-LDL. Figure 1B shows that hepatocytes cocultured with LSECs incorporated significantly more DiI-LDL than hepatocytes in other cultures. Only low-level fluorescence was detected in the other cocultures. Similar results were obtained in the cocultures of human hepatocytes and human LSECs (Fig. 1C). Figure 2A quantifies the LDL uptake in average fluorescence units. Hepatocyte-LSEC cocultures showed a 300% increase

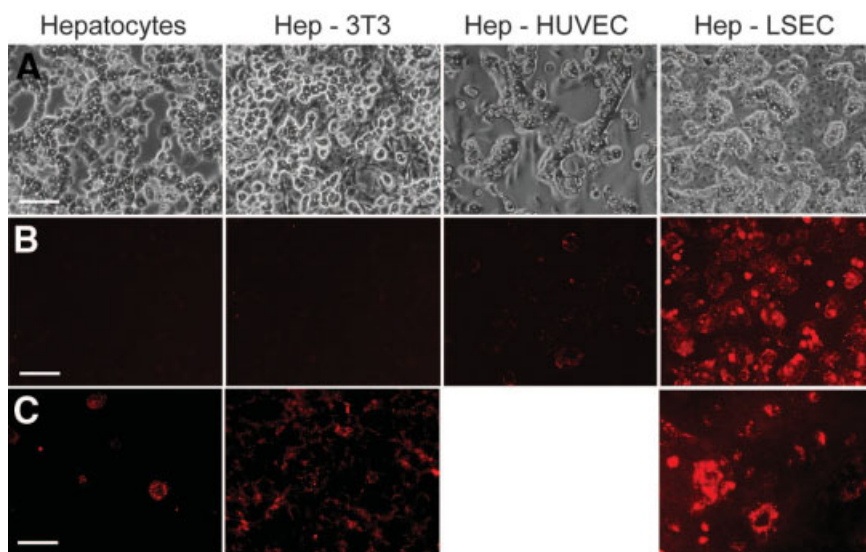


Fig. 1. LDL uptake in rat and human hepatocyte cultures and cocultures. (A) Phase contrast images of rat hepatocytes in a collagen double-gel culture, rat hepatocytes cocultured with 3T3-J2 fibroblasts, human umbilical vein endothelial cells, and rat LSECs. Bar = 100 μ m. (B) DiI-LDL uptake in rat hepatocyte cultures and cocultures after 3 hours incubation. Bar = 100 μ m. (C) DiI-LDL uptake observed in human hepatocyte cultures and cocultures with 3T3-J2 fibroblasts and human LSECs. Bar = 50 μ m. Hep, hepatocytes; HUVEC, human umbilical vein endothelial cells; LSEC, liver sinusoidal endothelial cells.

($\pm 20\%$; $P = .0008$; $n = 7$) in LDL uptake compared with hepatocytes in single culture or hepatocyte–3T3 cocultures. This result is surprising, because the coculture of hepatocytes with 3T3-J2 has been previously shown to express a wide range of liver-specific functions *in vitro*.^{23,24}

One possibility is that LSECs, not hepatocytes, take up DiI-LDL in coculture. To detect which cell type took up the LDL, we incubated the hepatocyte–LSEC cocultures in the presence of both DiI-LDL and FITC-acLDL. Figure 3 clearly shows that the hepatocytes specifically take up the DiI-LDL and are labeled red, while the LSECs specifically take up the acLDL, most likely through their scavenger cell receptor,²⁵ and thus are labeled green.

LDL Transcytosis. One possible mechanism for increased LDL uptake in hepatocyte–LSEC cocultures is transcytosis.²⁶ Recent studies suggest that endothelial cells transfer LDL across the blood–brain barrier through a caveolae-mediated process in a time scale of 10 to 20 minutes.²⁷ A similar interaction was suggested to occur in the liver.²⁶ To determine whether transcytosis occurs in our cocultures, we incubated the cells with DiI-LDL for 5, 10, and 15 minutes and immediately imaged the cells under fluorescence microscopy. Figure 4A–B shows that DiI-LDL was quickly taken up by hepatocytes after 15 minutes of incubation and not by LSECs as might have been expected from a transcytosis model. Similar results were observed following 5 and 10 minutes of incubation (data not shown).

To further investigate the possibility of transcytosis, we incubated the cocultures with filipin, a cholesterol-binding agent that causes the disassembly of caveolae.²⁷ The cells were pretreated with 3 μ g/mL filipin for 10 minutes at 37°C, then washed and incubated with DiI-LDL for 45

minutes. Although the LDL uptake following treatment with filipin appeared patchy, possibly due to membrane disruption, it was still localized within the hepatocytes in coculture while virtually no staining was found in the LSECs (Fig. 4C). These results further strengthen the hypothesis that LSECs induce LDL uptake in hepatocytes without involvement of transcytosis by the endothelial cells.

Role of LDL-R. LDL uptake *in vivo* is largely mediated by the hepatic LDL-R, expressed on the sinusoidal surface of hepatocytes. To determine if the increased uptake of LDL is mediated by the hepatic LDL-R, we incubated the cocultures with neutralizing antibodies against the LDL-R. Figure 2B shows DiI-LDL uptake in hepatocyte–LSEC cocultures incubated with 5, 15, and 25 μ g/mL antibody against the LDL-R. Neutralization of the LDL-R significantly reduced the uptake of LDL in culture ($P = .006$; $n = 3$). We note that the inhibition of the LDL-R only lowered the uptake to 55%, suggesting that other receptors, such as the closely related vLDL-R, might be involved in the LDL uptake as well.

Previous studies have shown that scavenger receptors expressed in hepatocytes might also contribute to LDL uptake.²⁸ To establish whether scavenger receptors are involved, we incubated the hepatocyte–LSEC cocultures with a fivefold excess of unlabelled acLDL (100 μ g/mL). Figure 2A shows that the addition of acLDL did not interfere with the internalization of DiI-LDL by hepatocytes in cocultures, suggesting that the hepatic scavenger cell receptors are not involved in the upregulation of LDL uptake.

Growth Factor-Mediated Uptake. The results above indicate that the increased LDL uptake in the hepatocyte–LSEC cocultures is mediated by the LDL-R. One possible

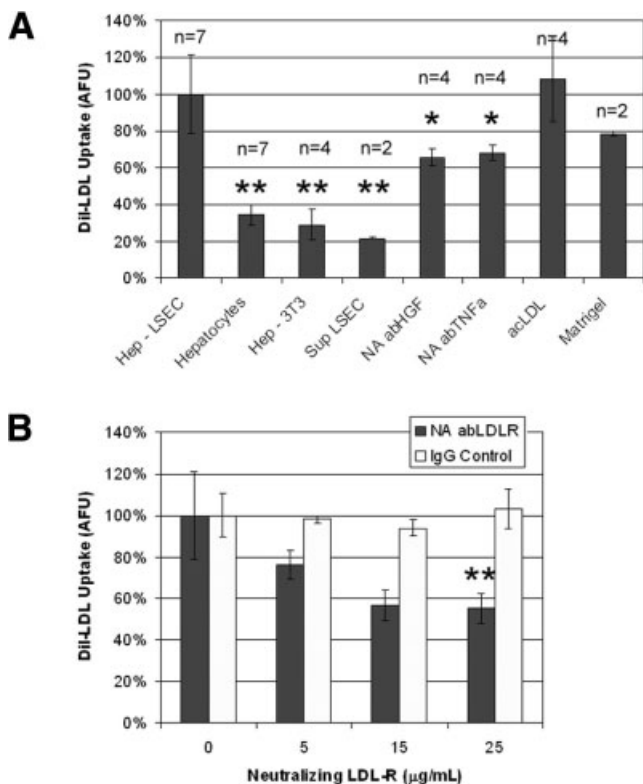


Fig. 2. LDL uptake in hepatocyte cultures and cocultures. Uptake was normalized to hepatocyte–LSEC cocultures from the same isolation cultured under identical conditions. (A) LDL uptake in hepatocyte–LSEC cocultures (Hep–LSEC), hepatocytes in single culture (Hepatocytes), hepatocyte–3T3 coculture (Hep–3T3), hepatocytes in single culture with supplement LSEC-conditioned medium (Sup LSEC), hepatocyte–LSEC cocultures with neutralizing antibodies against HGF (NA abHGF) or TNF- α (NA abTNFa), hepatocyte–LSEC cocultures incubated with 100 μ g/mL acLDL (acLDL), hepatocyte spheroids on Matrigel (Matrigel); n = number of experiments performed in duplicate. (B) LDL uptake in hepatocyte–LSEC cocultures in the presence of increasing concentration of neutralizing antibody against the LDL-R or control immunoglobulin G. * $P < .05$, ** $P < .01$ (significantly different from hepatocytes–LSECs). DiI-LDL, 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate-labeled LDL; AFU, average fluorescence units; Hep, hepatocytes; LSEC, liver sinusoidal endothelial cells; NA abHGF, neutralizing antibodies against hepatocyte growth factor; NA abTNFa, neutralizing antibodies against tumor necrosis factor α ; acLDL, acetylated LDL; NA abLDLR, neutralizing antibodies against low-density lipoprotein receptor; IgG, immunoglobulin G; LDL-R, low-density lipoprotein receptor.

explanation is that growth factors such as HGF or TNF- α secreted by LSECs in culture^{29–31} may upregulate the LDL-R expression in hepatocytes.^{18,19,32} We detected HGF in our LSEC cultures via immunofluorescence (data not shown) and TNF- α via ELISA (11 ± 4 pg/10⁶ cells/48 h). To determine whether these LSEC-secreted factors were responsible for the observed increase in hepatic LDL uptake, we incubated hepatocyte–LSEC cocultures with 25 μ g/mL neutralizing antibodies against HGF and TNF- α . DiI-LDL uptake in these cocultures was significantly decreased ($P = .014$, $n = 4$) and ($P = .045$, $n = 4$) by HGF and TNF- α neutralization, respec-

tively (Fig. 2A). However, hepatocytes in single culture incubated with 100 ng/mL of either HGF or TNF- α did not exhibit an increased uptake of LDL (data not shown), suggesting that other factors mediate the interaction between LSECs and hepatocytes.

LSECs Mediate LDL-R, EGF-R Expression. Hepatocytes in the normal liver exhibit a polarized morphology characterized by the division of the plasma membrane into sinusoidal, lateral, and apical domains.³³ Previous studies have shown that whereas hepatocyte spheroids formed on Matrigel exhibited all three surfaces,³⁴ hepatocytes cultured in a collagen double-gel did not express the EGF-R commonly found on the sinusoidal surface of hepatocytes.³⁴ Interestingly, hepatocyte spheroids on Matrigel exhibit a high level of DiI-LDL uptake similar to hepatocyte–LSEC cocultures (Figs. 2A, 5).

To discern whether LSECs induce hepatocytes to exhibit a sinusoidal surface (the appropriate receptors), we stained our cultures and cocultures against the LDL-R and the EGF-R. Figure 6 shows that hepatocytes cultured in a collagen double-gel configuration or cocultured with fibroblasts display only a low level of the LDL-R and EGF-R. On the other hand, hepatocytes cocultured with LSECs under the same conditions show a high level of LDL-R and EGF-R. Similar staining of LDL-R and EGF-R was found in hepatocyte spheroids formed on Matrigel.

Reverse-transcriptase polymerase chain reaction analysis shows that hepatocyte–LSEC cocultures express a higher level of LDL-R and EGF-R messenger RNA compared with hepatocytes in single culture (Supplementary Fig. 1: Supplementary material for this article can be found on the HEPATOLOGY website (<http://interscience.wiley.com/jpages/0270-9139/suppmat/index.html>)). These gene expression data are consistent with our previous results regarding LDL uptake (Fig. 2) and LDL-R protein expression (Fig. 6) in hepatocyte–LSEC cocultures.

Uptake of HCV Heterodimer (E1, E2)-Coated Beads in Culture. Several studies have suggested a role

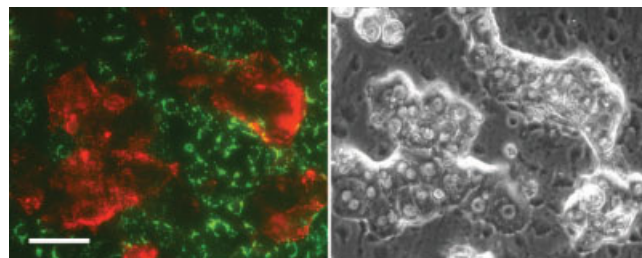


Fig. 3. Uptake of DiI-LDL and FITC-acLDL in hepatocyte–LSEC cocultures. The left panel shows DiI-LDL (red) and FITC-acLDL (green) labeling distribution; the right panel shows the corresponding phase contrast image. Bar = 50 μ m.

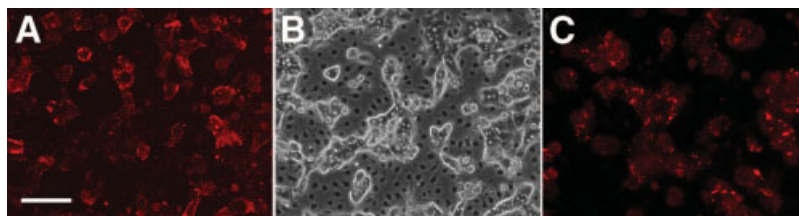


Fig. 4. (A) Uptake of LDL in rat hepatocyte-LSEC cocultures after 15 minutes' incubation with Dil-LDL. (B) Phase contrast image of the same coculture. (C) LDL uptake in rat hepatocyte-LSEC cocultures after 10 minutes' incubation with 3 $\mu\text{g}/\text{mL}$ filipin, a caveolae inhibitor. Bar = 100 μm .

for the LDL-R in HCV uptake.^{8,35} In addition, L-SIGN, a liver-specific receptor for HCV, was found to be expressed on LSECs.³⁶ Therefore, our hepatocyte-LSEC cocultures might be uniquely suited for the study of HCV infection. To study the interaction between HCV E1 and E2 and our hepatocyte-LSEC cocultures, we expressed and purified a properly folded E1 and E2 heterodimer as described by Cocquerel et al.²¹ The heterodimer was then linked to 100-nm-diameter fluorescent beads, roughly the size of the virus (≈ 60 nm).³⁷ The E1- and E2-linked bead suspension was incubated with hepatocytes in the presence or absence of LSECs for 3 hours. Figure 7 shows that whereas hepatocytes in single culture on collagen did not take up the E1- and E2-linked beads, hepatocytes in cocultures took up a high level of E1- and E2-linked beads. Blocking the LDL-R by incubating with neutralizing antibodies reduced the uptake of E1- and E2-linked beads by the hepatocytes in coculture. The LSECs in coculture took up E1- and E2-linked beads regardless of the presence of neutralizing antibodies, possibly due to an L-SIGN like receptor³⁶ or a nonspecific scavenger receptor.²⁵ Fluorescent beads without the E1 or E2 heterodimers were not taken up by either culture. These results suggest that hepatocyte-LSEC cocultures might be suited for the study of HCV infection *in vitro*.

Discussion

The parenchymal cells of the liver play an important role in cholesterol metabolism, producing vLDL, clearing oxidized LDL, and removing excess LDL from the blood.^{2,25,33} However, studies on LDL uptake in cultured hepatocytes were mostly limited to nonpolarized cell lines such as HepG2, because LDL-R expression is lost in primary cultured hepatocytes.^{6,18,19} The LDL-R has also been shown to play a role in HCV infection.⁶ Wild-type HCV particles were found to be associated with LDL and vLDL in blood,⁶⁻⁸ suggesting that the virus might "hitch a ride" into hepatocytes by binding to LDL. Loss of LDL receptor expression in primary cultured hepatocytes might account for the difficulty of infecting the cells with wild-type HCV (bound to LDL) *in vitro*.^{38,39}

Our results demonstrate that established culture techniques such as the collagen double gel and coculture with fibroblasts that express long-term stable function are inadequate for the study of LDL metabolism or HCV infection due to loss of sinusoidal-specific receptors. On the other hand, high levels of the LDL-R and the EGF-R can be displayed by hepatocytes when cocultured with LSECs on a single layer of collagen. This technique is simple and relies on native cells isolated from the nonparenchymal section of the liver.

The mechanism through which liver endothelial cells induce the expression of hepatic sinusoidal receptors is unknown.⁴⁰ Our time-lapse and caveolae inhibition results (Fig. 4) suggest that LDL transport between the cells does not play a role in the hepatocellular uptake of LDL, because dye dissolved in the cholesterol core of DiI-LDL did not stain the LSECs at any time point examined, even after several hours of incubation. One intriguing possibility is that LSECs stimulate hepatocytes to secrete lipoproteins or produce bile acids, depleting the cholesterol stores in hepatocytes, thus inducing the expression of LDL-R and the uptake of LDL.^{32,40} Such stimulation could occur through HGF, TNF- α , or other mediators.^{18,19,32} However, this hypothesis does not explain the expression of the EGF-R in hepatocyte-LSEC cocultures.

Another possibility is that cell-cell contacts through a membrane-associated component (*e.g.*, heparan sulfate proteoglycan) could signal hepatocytes to sequester a si-

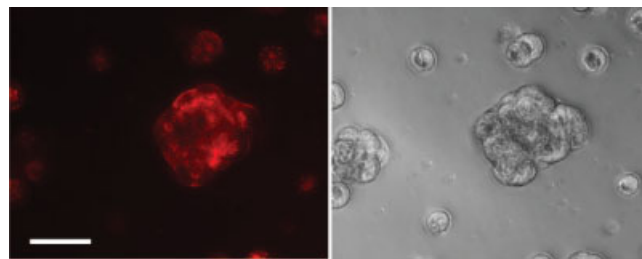


Fig. 5. Uptake of DiI-LDL by a hepatocyte spheroid on Matrigel. The left panel shows DiI-LDL (red) distribution; the right panel shows the corresponding phase contrast image. Bar = 50 μm .

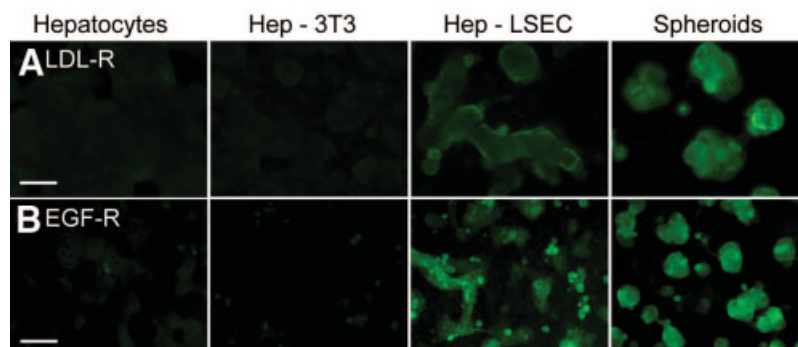


Fig. 6. Immunofluorescence staining of LDL-R and EGF-R in cultured hepatocytes. (A) LDL-R distribution in hepatocytes in single culture, hepatocytes cocultured with 3T3-J2 fibroblasts or rat LSECs, and hepatocyte spheroids on Matrigel. Staining of LDL-R is primarily localized to the hepatocyte–LSEC interface in the cocultures and hepatocyte edges in the spheroids on Matrigel. The apparently higher intensity in spheroids versus LSEC cocultures is due to hepatic aggregation, because average signal intensity was similar. Bar = 50 μm . (B) Staining for the EGF-R follows a similar pattern. Bar = 100 μm . Hep, hepatocytes; LSEC, liver sinusoidal endothelial cells; LDL-R, low-density lipoprotein receptor; EGF-R, epidermal growth factor receptor.

sinusoidal domain on which the LDL-R and EGF-R are expressed.^{16,34,41} Heparan sulfate proteoglycan is a major component of the liver basement membrane, which is synthesized by LSECs but not by hepatocytes or liver fibroblasts.⁴² In addition, this proteoglycan is naturally found in Matrigel but not in collagen type I, suggesting that it might be picked up during spheroid formation.⁴³ Future studies on the interaction between endothelial cell-secreted proteoglycan and hepatocytes *in vitro* are warranted.

Based on the current and previously published results, a hypothetical model for hepatic polarization in

different culture configurations is summarized in Fig. 8. Hepatocytes cultured in a collagen double-gel have been shown to exhibit native cell–cell contacts such as E-cadherin and bile canaliculi,³⁴ but do not express sinusoidal receptors such as EGF-R³⁴ and LDL-R. Hepatocytes cocultured with 3T3 fibroblasts exhibit hepatic cell–cell contacts such as connexin-32⁴⁴ and bile canaliculi,²⁴ but also do not express the EGF-R and LDL-R. On the other hand, hepatocytes cocultured with LSEC show both traditional polarity markers,^{45,46} and express a high level of sinusoidal receptors (EGF-R, LDL-R) at the interface between the hepatocytes and the LSEC. At least part of the interaction between hepatocytes and LSECs has been shown to be mediated by growth factors.^{29,47} Similarly, hepatocyte

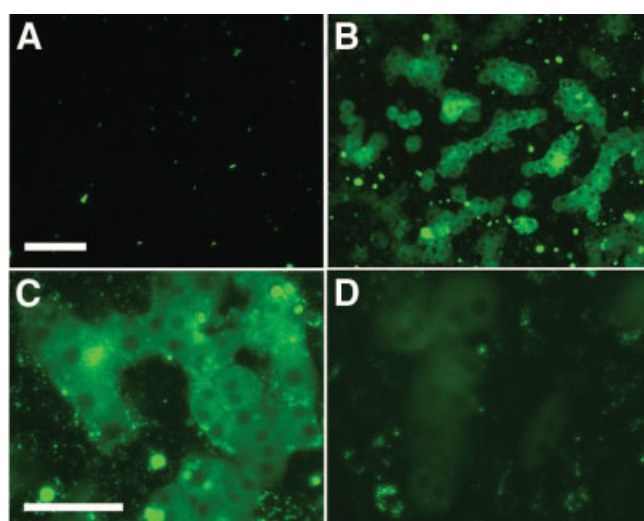


Fig. 7. Uptake of fluorescent E1E2-linked beads in hepatocyte cultures and cocultures after 3 hours of incubation. (A) Hepatocytes in single culture on collagen gel. (B) Hepatocytes in coculture with LSECs. Bar = 100 μm . (C) High-magnification image of hepatocyte–LSEC cocultures. Beads are both inside and on the periphery of the hepatocytes. Labeling observed on dark background is by LSECs. (D) Conditions similar to panel C, except that 25 $\mu\text{g}/\text{mL}$ neutralizing antibody against LDL-R was present. Bar = 50 μm .

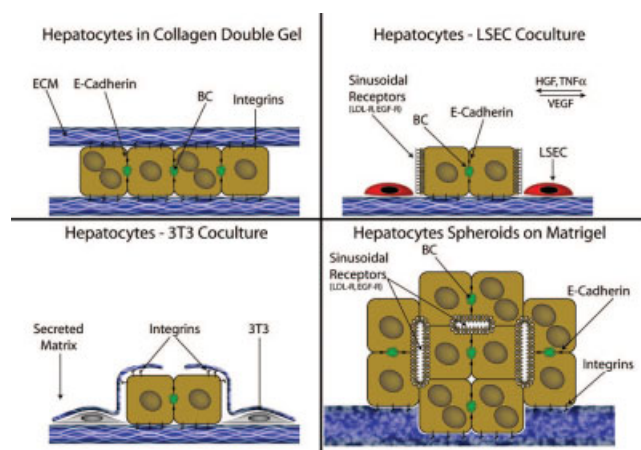


Fig. 8. Schematic of a hypothetical model showing the polarization of hepatocytes in different culture configurations. ECM, extracellular matrix; BC, bile canaliculi; LSEC, liver sinusoidal endothelial cells; LDL-R, low-density lipoprotein receptor; EGF-R, epidermal growth factor receptor; HGF, hepatocyte growth factor; TNF- α tumor necrosis factor α ; VEGF, vascular endothelial growth factor.

spheroids were shown to express E-cadherin,³⁴ form extensive bile canaliculi,⁴⁸ and show sinusoidal surface markers at the interface between cells in the spheroid.⁴⁸

In conclusion, cocultures of LSECs and hepatocytes express high levels of LDL-R and therefore could be used for the study of LDL uptake and clearance by primary hepatocytes. In addition, we hypothesize that similar cocultures might be permissive to the hepatitis C virus, which was shown to interact with the LDL-R,⁶ CD81,⁸ and the liver-specific L-SIGN receptor expressed on LSECs.³⁶

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