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3-D Coculture of Hepatic Sinusoidal Cells with Primary Hepatocytes—Design of an Organotypical Model

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Models for cocultures of parenchymal (PC) and nonparenchymal cells (NPC) of the liver relied on mixing the cells in a two-dimensional configuration or on establishing spheroidal aggregates. *In vivo* hepatic nonparenchymal cells, such as endothelial cells and Kupffer cells, are separated from parenchymal cells by extracellular matrix (ECM). Due to their location outside of the space of Disse they can form a barrier toward the sinusoid. Hepatocytes are attached to ECM of the space of Disse via two opposing sinusoidal surfaces. No three-dimensional coculture model reflecting this specific microenvironment of the liver cell plates *in vivo* has been available to date. We designed a three-dimensional model by positioning NPC on top of PC enclosed as a monolayer within a collagen sandwich. A gas-permeable membrane support can be used to allow the supply of oxygen to the resulting cell plate also from underneath the cell layers. Morphological analysis was performed by inverse and cross-sectional studies by light microscopy, scanning, and transmission electron microscopy of the coculture model. Cuboidal hepatocytes formed confluent layers below the NPC layer. They regularly expressed bile canaliculi at intercellular contact zones. Both sinusoidal surfaces expressed microprojections. Characteristic NPC including endothelial cells, Kupffer cells, and Ito cells completely covered the second matrix layer within a week. Kupffer cells were located on top of endothelial cells. Ito cells were intermingled and could be identified by their intracytoplasmic lipid droplets. LPS stimulation of cocultures resulted in a depression of albumin secretion. Phase I and phase II metabolites of the cytochrome P-450 1A1 substrate ethoxyresorufin were generated independently from the presence of cocultured NPC. This study describes the development of a novel three-dimensional coculture model, which intends to mimic more closely the microenvironment of the hepatic sinusoid by respecting the specific plate structure of the liver parenchyma. The model could serve as a complex tool to study potential collaborations between PC and NPC of the liver.

INTRODUCTION

The liver is an organ of considerable cellular heterogeneity. The structural complexity has become evident by the degree of interactions that occur between the parenchymal (PC) and the nonparenchymal cells (NPC). The latter include Kupffer, pit, Ito, and endothelial cells.

Following a period of studies on isolated PC or NPC, more studies on cocultures of PC with NPC need to be initiated. This approach will further enhance our knowledge about the physiological role of the various components of the liver cell populations [1] and the relevance of their structural organization and collaboration *in vivo*.

No coculture model of PC and NPC that intends to mimic the characteristic three-dimensional relationship of these cells within the microenvironment of the liver trabecles and hepatic sinusoids has been available to date. Besides these structural considerations, functional and morphological stability of the parenchymal liver cells *in vitro* has been a limiting factor. Indeed, coculture models were designed to support hepatocellular functions in two-dimensional culture configurations [2, 3]. Coculture models between PC and NPC of the liver have so far mostly been limited to mixing the cells in a monolayer configuration with one-sided attachment to extracellular matrix (ECM) only [4–7]. This represents a classic approach for the hepatocyte culture but permits only unilateral attachment to extracellular matrix. In an attempt to improve cellular interaction, cocultures were established by forming multicellular spheroids [8]. Leakage of GOT and LDH in the latter model was more pronounced than in a conventional single gel monolayer culture configuration [8].

In vivo hepatocytes are enclosed within the ECM of the space of Disse. The space of Disse provides anchorage for hepatocytes and hepatic sinusoidal cells. It is a

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key feature that the space of Disse is present on two opposing sinusoidal surfaces enclosing the hepatocyte. Endothelial cell surfaces are either directed toward the sinusoid or anchored to the underlying ECM (Fig. 1A). Kupffer cells are located on top of endothelial cells, and the "perisinusoidal" cells or Ito cells can be found also inside the space of Disse extending processes toward the hepatocytes. Pit cells are considered resident liver cells and always found in the lumen of sinusoids [9, 10]. Sinusoids are dilated vascular rooms inside the liver carrying a mixture of portal venous and arterial blood. Nutrients, toxins, and hepatocellular secretory products cross the nonparenchymal cell layer and the space of Disse. The cellular lining of the sinusoids is thought to constitute a coordinate defence system protecting hepatocytes against injury [11]. Kupffer and endothelial cells participate in phagocytosis [11]. Perisinusoidal cells are involved in lipid and vitamin A metabolism [11]. Pit cells have been described as large granular lymphocytes and express natural killer (NK) activity. There is broad evidence of intercellular communication between NPC and PC by, e.g., prostaglandins [11–13]. Nitric oxide is released in response to bacterial endotoxin in Kupffer cell hepatocyte cocultures [5]. NPC and PC act as functional units and communication as well as collaboration seems important for the differentiated function of the liver [5, 9]. Coculture of NPC with PC enhanced the stability of NPC to express insulin-like growth factor [7]. A protein produced by NPC induces PC proliferation [1]. Cooperation of NPC with PC was suggested for purine metabolism [14]. NPC contribute to the metabolism of xenobiotics [15, 16].

Hepatocytes *in vivo* perform several drug oxidation and conjugation reactions. Multiple forms of cytochrome P-450 (CYP), a family of monooxygenases, catalyze phase I biotransformation reactions of a large variety of xenobiotics, including drugs and endogenous substrates [17]. Oxidation by monooxygenases provides a site on the molecule for subsequent phase II conjugation to sulfate and glucuronic acid [18, 19].

In order to mimic more closely the microenvironment of the hepatic sinusoid we designed an organotypical three-dimensional coculture model between PC and NPC. This model utilizes primary matrix overlaid hepatocytes (MOH). MOH have been shown to retain differentiated functions [20, 21]. Functional stability has been demonstrated for protein secretion, transcription [20], and drug biotransformation [22, 23]. We have previously shown that MOH are capable of autoregulatory control of an acute phase response upon exposure to interleukin-6 [21].

The coculture model is established by adding liver NPC on top of MOH in a three-dimensional configuration. A gas-permeable membrane (Fig. 1B) can be used as a mechanical support. Use of such a membrane permits oxygen supply to the resulting cell–matrix plate from a second side. In standard culture dishes oxygen is supplied only via the supernatant.

This study presents ultrastructural evidence of intact three-dimensional cocultures of NPC with matrix overlaid primary rat hepatocytes. Collaboration between both cell populations is demonstrated following lipopolysacharide (LPS) stimulation and consecutive changes in the albumin secretory pattern of hepatocytes. Maintenance of phase I and II metabolism with and without cocultures is shown for the CYP 1A1 substrate exthoxyresorufin.

METHODS

Cell isolation. Cells were isolated according to the method described by Seglen [24] using female Wistar rats weighing 200–250 g. The cell suspension obtained was purified by means of Percoll (Pharmacia, Freiburg, Germany) gradient centrifugation at 50*g* for 5 min at 4°C. For this purpose 12.5 ml cell suspension in Krebs–Ringer buffer (KRB) was mixed with 1.2 ml of Hanks' balanced salt solution and 10.8 ml of Percoll. Viability of the PC ranged between 85 and 98% as assessed by trypan blue exclusion.

Sinusoidal cells were isolated from the supernatant obtained by centrifugation at 19*g* (4°C for 5 min) of the initial cell suspension obtained by collagenase digestion of the liver. The supernatant was centrifuged at 813*g* for 10 min at 4°C to obtain a cell pellet, resuspended in 40 ml of KRB, and centrifuged again at 813*g* for 10 min at 4°C. The pellet was again resuspended using 20 ml KRB. For gradient centrifugation 5 ml of the resulting cell suspension was layered on top of 7 ml KRB containing 2 g metrizamide (Sigma, Deisenhofen, Germany). On top 1 ml of KRB was placed. This column was centrifuged at 1850*g* for 10 min at 4°C. The total fraction of NPC obtained by this procedure was used for establishing the cocultures.

Preparation of cocultures. Freshly isolated hepatocytes were seeded at a density of 3×10^6 cells per dish (28 cm^2) on collagen-coated Teflon membranes. Petriperm petri dishes were purchased from Bachofer (Frankfurt, Germany). One hour following attachment, the hepatocytes were overlayed with a second layer of collagen. One hour following gelation of this second layer, NPC were seeded at a density of 4×10^5 cells/ml on top of this second layer of collagen. PC and NPC originating from the same isolation were used for establishing cocultures. Experiments were repeated four times using cells from different animals. Four dishes were prepared each time either for cocultures or for controls without cocultured NPC. Cells were maintained in culture for 1 week.

Matrix and culture medium. Rat tail collagen was prepared according to the method described by Elsdale and Bard [25]. A final concentration of 1.11 mg/ml collagen was used for coating. Culture medium was collected daily and used for protein analysis. The pH of the collagen was adjusted to 7.4 immediately before coating, using a 10× Dulbecco's modified Eagle's medium (DMEM) concentrate (Biochrom, Berlin, Germany). For each collagen layer a volume of 1 ml was used.

Williams E supplemented with 10% fetal bovine serum (Biochrom), prednisone 9.6 μg/ml, glucagon 0.014 μg/ml (Novo, Mainz, Germany), and insulin 0.16 U/ml (Hoechst, Frankfurt, Germany) was used as culture medium. Glutamate and penicillin/streptomycin (Biochrom) were added. Culture medium (2 ml) was replaced daily and stored a 4°C for further analysis.

Morphological analysis. For light, transmission, and scanning electron microscopy the cells were fixed on the culture dishes by immersion with glutaraldehyde (2.5%, in 0.1 mol/liter cacodylate buffer, pH 7.4) immediately after removal of the culture medium. Fresh rat whole liver specimens were cut in pieces of 1×1 mm and were fixed in the same manner. All specimens were postfixed with

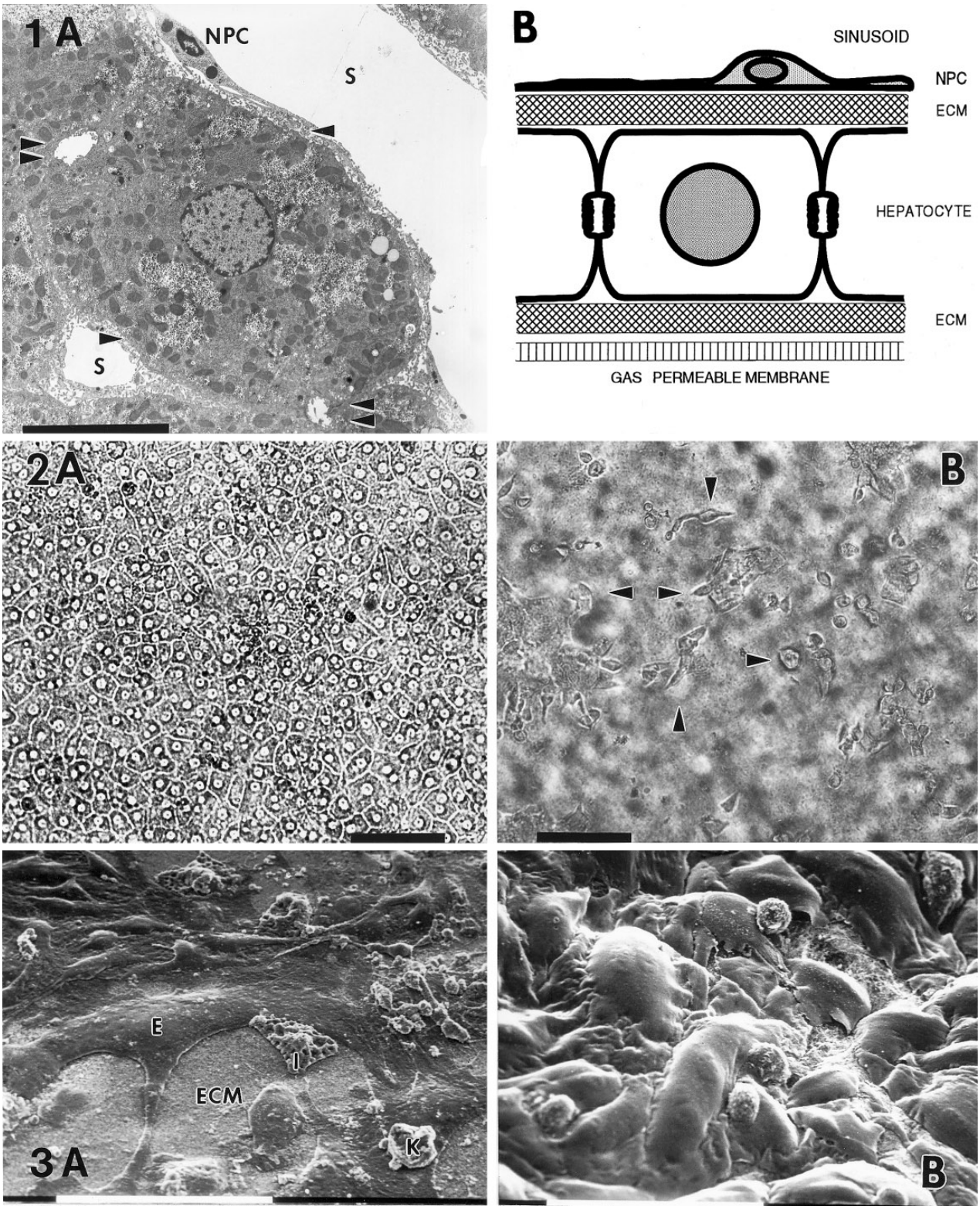


FIG. 1. (A, B) Micrograph of normal rat liver (A), showing a cuboidal hepatocyte enclosed within the space of Disse (arrowheads), sinusoids (S), and a nonparenchymal cell (NPC) in the typical morphology of an endothelial cell. On each side of the hepatocyte a cross-section of a bile canaliculus (double arrowheads) is seen. Bar, 5 μm. A schematic drawing of the three-dimensional coculture model is shown in B. Hepatocytes are layered on a gas-permeable membrane covered by a first, inferior, layer of hydrated collagen. NPC are placed on top of a second, superior, layer of collagen covering the hepatocytes and facing the supernatant.

FIG. 2. (A, B) MOH on gas-permeable membranes at 2 days in culture forming a confluent layer (A). Bile canaliculi (lucent rim) surround each individual hepatocyte. (B) NPC (arrowheads) are located on top of the MOH, which can be seen in the background out of focus. NPC constitute a heterogeneous population and are loosely arranged 48 h after seeding. Bar, 100 μm.

FIG. 3. (A, B) Scanning electron microscopic picture 2 days after seeding (A). NPC only partially cover the superior collagenous matrix layer (ECM). Different cell shapes referring to Kupffer (K), endothelial (E), and Ito (I) cells can be distinguished. NPC at 7 days (B) in culture have become confluent and almost completely cover the superior collagen layer. Bar, 100 μm.

2% OsO_4 in the same buffer for 1 h and dehydrated in graded alcohol solutions. Areas of approximately 4 × 10 mm surface containing cells and the respective collagen layers were cut out of the culture dishes and embedded in epoxy resin (Epon). Sections were cut orthogonally to the cell layer showing the cells in cross section. For light microscopy (LM) semithin sections were stained with alkalinized toluidine blue. For scanning electron microscopy (SEM) the culture specimens were repeatedly treated with osmium tetroxide (OTOTO-method), dehydrated in graded acetone solutions, and critical point dried over CO_2. Then they were mounted on specimen holders with conductive paint and examined in a Philips 505 scanning electron microscope. For transmission electron microscopy (TEM) ultrathin sections of epoxy resin-embedded cultures and whole liver specimens were stained with uranyl acetate and lead citrate and observed in a ZEISS EM 10 electron microscope.

Albumin secretion and LPS stimulation. Albumin secretion was measured in the supernatants of cocultures between NPC and MOH by immunometric methods (ELISA). Groups consisting of MOH without cocultures were used as controls. Chromatographically purified rat albumin and the monoclonal antibody for rat albumin were purchased from Cappel (Durham, NC). Ninety-six-well plates (Nunc, Wiesbaden, Germany) were coated with PBS containing 1 mg/ml of albumin and left overnight at 4°C. After washing the plate four times with PBS–Tween, 50 μl of cell culture supernatant was added to each well. Thereafter, wells were incubated with 50 μl of horseradish peroxidase-conjugated anti-rat albumin antibody. Following an incubation period of 24 h at 4°C, substrate buffer containing *O*-phenylenediamine dihydrochloride and H_2O_2 (Sigma) was added for 6 min. The reaction was stopped with 100 μl of 8 *N* H_2SO_4. Absorbance was measured at 490 nm using a Dynatech M5000 ELISA plate reader.

For assessment of the albumin secretory pattern following LPS stimulation, hepatocytes were seeded on collagen-coated tissue culture polysterene 6-well plates (Nunc, Wiesbaden, Germany) at 80,000 cells/cm^2. NPC were seeded on top of the second collagen layer as described above. One milliliter of culture medium was replaced every 24 h. FITC-LPS from *Escherichia coli* (Sigma) was added at a concentration of 200 μg/ml to the supernatant for 24 h starting on the sixth day in culture. Supernatants were collected daily and frozen at −20°C for analysis of albumin. Cultures were maintained for 16 days.

7-Ethoxyresorufin O-deethylation (EROD) assay. The O-deethylation of 7-ethoxyresorufin was measured by a modification [26] of assays developed by Burke and Mayer [27] and Klotz *et al.* [28].

Hepatocyte cultures and cocultures form three different isolations were incubated with 3 μmol/liter 7-ethoxyresorufin (Sigma Chemie GmbH, Deisenhofen, Germany) and 10 μmol/liter dicoumarol (Aldrich, Steinheim, Germany). Aliquots of the supernatant medium were withdrawn after 1 h and frozen immediately in liquid nitrogen. Formation of resorufin was quantified by HPLC analysis using a HP 1090 M (Hewlett–Packard, Waldbronn, Germany) equipped with a spectrofluorimeter (HP 1046 A, Hewlett–Packard) with an excitation wavelength of 540 nm and the emission wavelength of 586 nm. The spectrofluorimeter was calibrated using resorufin standards. Resorufin conjugates were cleaved using β-glucuronidase and arylsulfatase from *Helix pomatia* (Serva, Weinheim, Germany) in 100 mmol/liter acetate buffer (pH 4.66) for 12 h at 37°C. HPLC was performed with a LiChrospher 100 RP 18 column (250 × 4 mm i.d., particle size 5 μm endcapped, E. Merck, Darmstadt, Germany) and a LiChrospher 100 RP 18 guard column (4 × 4 mm i.d., particle size 5 μm). Gradient elution was carried out at a flow rate of 1.3 ml/min. The mobile phase consisted of a 50 mmol/liter ammonium acetate buffer, pH 7 (solvent A) and acetonitrile. The operating conditions were started with 100% A for 6 min, changed to 60% A after 15 min, isocratic elution for 2 min, then changed to 50% A after 20 min, and to 0% A after 30 min.

Immunoblot analysis. O-deethylation of 7-ethoxyresorufin is related to the immunoblot analysis expression of cytochrome P450 1A1 [27]. Expression of cytochrome P450 1A1 enzyme in cocultures was therefore also analyzed by Western blot. Controls consisted of matrix overlaid hepatocytes without an additional layer of NPC. Hepatocellular cytochrome P450 1A1 contents were assessed on Days 1, 3, and 7 in culture. For separating the cells from the matrix layers, culture dishes were incubated for 30 min with 4 ml phosphate-buffered saline (PBS) containing 1 mg/ml collagenase (Sigma). After digestion of the collagen matrix, the resulting cell suspension was aspirated and centrifuged at 50*g* for 5 min. Low-speed centrifugation resulted in a pelleting of hepatocytes, while the smaller NPC remained in the supernatant. The remaining cell pellet was washed twice to further separate the PC form the NPC. The pellet was frozen at −20°C. For cell homogenate preparation of the hepatocytes, the cells were centrifuged at 350*g* for 10 min at 4°C. For washing, the cell pellet was suspended in Tris-buffered saline (TBS), pH 7.6, and at 4°C and centrifuged again. This procedure was repeated twice. The cells were finally suspended in 300 μl double-distilled H_2O and homogenized with a Branson sonicator (15 s, 40 W). The extent of cell disruption was assessed microscopically to be >90%. Protein concentrations were determined using the bicinchoninic acid (BCA) method with bovine serum albumin (BSA) as standard [29]. Electrophoresis was done using a 7.5% polyacrylamide stacking gel and a 10% SDS–polyacrylamide resolving gel, according to the method of Laemmli *et al.* [30]. For blotting a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA) and a semidry blotter (Biometra, Göttingen, Germany) were used. Cytochrome P450 1A1 protein was detected using an anti-cytochrome P450 1A1 polyclonal rabbit antibody (Oxygene, Dallas, TX) and a biotinylated donkey anti-rabbit immunoglobulin (Amersham, Braunschweig, Germany) with a streptavidin–biotin–alkaline phosphatase conjugate. Blots were stained with a 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitroblue tetrazolium (NBT) solution (Sigma). Freshly prepared rat liver microsomes were used as a control for the antibody to detect cytochrome P450 1A1. The experiment was repeated three times with cells from three different isolations. Equal amounts of protein (10 μg) were used for analysis.

RESULTS

Morphology

We used inverted light microscopy of the three-dimensional coculture model to investigate the different cell layers. At the inferior cell layer (Fig. 2A) hepatocytes after 2 days had constituted a relatively homogeneous population of polyhedric cells with large round nuclei. Each cell was surrounded by a lucent rim which represented the bile canaliculus (Fig. 2A). In a manner typical for cultures on gas-permeable membranes, attachment resulted in the formation of confluent hepatocellular monolayers with 1.07×10^5 cells/cm^2. Without a membrane the hepatocellular density was 0.71×10^5 cells/cm^2. After 7 days in culture, the hepatocytes had maintained their cuboidal cell shape and height. Their nuclei were still roundish and the bile canaliculi were present in regular position with unchanged frequency between the cells. Occasionally the bile canalicular diameter was slightly increased.

After 2 days in culture (Fig. 2B) a heterogeneous population of NPC had developed on top of the superior layer of matrix. PC and NPC were distinguished by their different cell shapes. The NPC were smaller than PC and had a highly variable cell shape (Fig. 2B). In the background of the latter figure, PC were faintly detectable being out of the focus plane. Cells located

on top of the second matrix layer appeared blurred, since they were seen across the confluent MOH layer (Fig. 2B).

The different cell shapes of NPC, which were still only partially covering the superior matrix layer after 2 days in culture, could be identified more clearly in SEM (Fig. 3A). The cell population included flat polygonal cell bodies, belonging to endothelial cells. A central elevation represented the nucleus. Round cells represented Kupffer cells. Angular appearing cells with indented and elevated portions of the cytoplasm were Ito cells. Kupffer cells were found located on top of endothelial cells. Some areas of the surface were not covered by cells. In such locations the hydrated collagen layer could be seen exposed (Fig. 3A). At 7 days (Fig. 3B), NPC had formed confluent layers densely covering the collagen surface. The cell population was still heterogeneous.

In light microscopy of cross sections (Figs. 4A and 4B) a heterogeneous and loosely arranged population of NPC was seen. Isolated flat cell bodies stretched out on the ECM with occasional elevation caused by the nucleus and the perinuclear zone. Kupffer cells were interspersed between or located on top of the endothelial cells (Fig. 4B). The thickness of the matrix layer covering the hepatocyte was about 50–200 μm. MOH constituted a monolayer of relatively uniform cells with large round nuclei and polyhedric cell shapes after 2 and 7 days in culture (Figs. 4A and 4B). In TEM an overview cross section (Fig. 5, Day 1) reveals round-shaped NPC with dark cytoplasmic inclusions. At high resolution (Fig. 7A, Day 1) these cells were identified as Kupffer cells having large cytoplasmic extensions like filopodia and lamellopodia on their surface. These cells also expressed numerous organelles and phagolysosomes. Frequently the Kupffer cells were found lying on long cytoplasmic expansions of endothelial cells (Fig. 5). Endothelial cells constituted the majority of the flat-shaped cells and showed a homogeneous cytoplasm without lucent inclusions and only few and small dense inclusions. Figure 7B shows a detail of an endothelial cell body with an elongated nucleus. In comparison to the Kupffer cells these cells have relatively smooth cell surface devoid of microprojections. At the surface facing the supernatant stress fibers are found, coated pits toward the ECM. Ito cells could be identified by the prominent lipid inclusions (Fig. 7C). They were often intercalated between other cells. Glycogen particles were scattered or found in minute clusters. A few caveolae could be present along the plasma membrane (Fig. 7C). Occasionally cytoplasmic areas were lying underneath the superficial endothelial cell layer in a spot-like or layer-like arrangement (Fig. 5). Cytoplasmic expansions could be detected radiating from these cells (Fig. 5). Pit cells were not detected.

By Day 7 PC still had a cuboidal shape and distinct sinusoidal and biliary surfaces (Fig. 5). Bile canaliculi were regularly present. At higher magnification hepatocytes were characterized by large roundish nuclei (Fig. 6). Their sinusoidal cell surfaces were not smooth like the surfaces of endothelial cells (Fig. 7B), but rather exhibited numerous cytoplasmic microprojections (microvilli and microplicae) as *in situ*. These structures were directed toward both sides flanking the two sandwich like collagen layers (Fig. 6). The cytoplasmic components of the hepatocytes were morphologically unchanged and comparable to an *in situ* state.

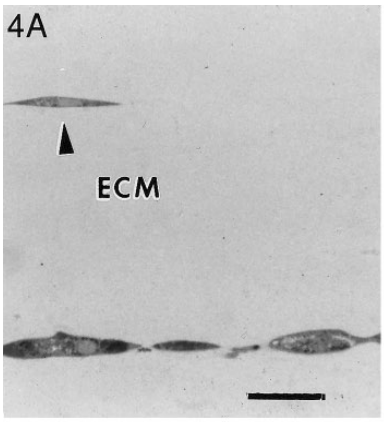
Albumin secretion and LPS stimulation. Albumin secretion was studied with and without the presence of nonparenchymal cells. Albumin secretion averaged for the first 24 h in culture was 3.47 ± 0.92 μg/h/3×10^6 cells. After 7 days MOH characteristically had an increased albumin secretion reaching 120% respective to the postisolation rate. MOH in coculture with NPC showed a parallel behavior with increased albumin-secretion at Day 7. No significant difference with regard to albumin secretion was found for both groups (data not shown).

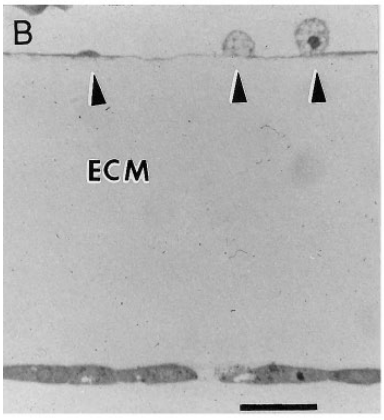
Regulation of albumin secretion during an acute phase response after addition of LPS to the supernatant is shown in Fig. 8. Three-dimensional cocultures exposed to LPS from *E. coli* reduced albumin secretion to 65% of the prestimulatory value upon a 24-h exposure on Day 6 in culture. The LPS-exposed group reached its minimal level 2–3 days following exposure to LPS. Four to five days following exposure to LPS, albumin secretion was gradually increased.

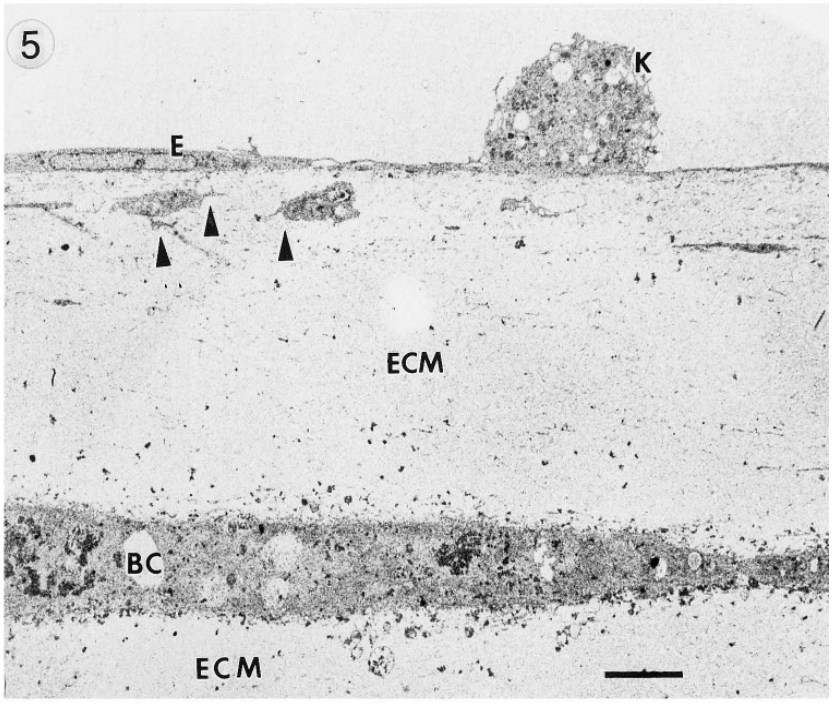
Biotransformation of ethoxyresorufin. No difference was observed in phase I and phase II activities of cocultured hepatocytes relative to the controls without the presence of NPC. EROD activity was slightly increased to about 140% at Day 7 relative to the activity at Day 3 (Fig. 9A). The metabolite pattern showed the same proportion of free resorufin to its phase II conjugates at Day 3 and Day 7 (Fig. 9B). Cytochrome P450 1A1 contents in MOH with and without cocultured NPC at Days 1, 3, and 7 were analyzed by immunological methods (Western blot, Fig. 9C). Both groups maintained P450 1A1 expression for at least 7 days.

DISCUSSION

The study describes the three-dimensional coculture of hepatic sinusoidal cells with matrix overlaid parenchymal liver cells. This novel *in vitro* model aims at imitating the *in vivo* microenvironment of the liver. In this coculture setting, hepatocytes typically maintained expression of sinusoidal surfaces with microprojections on both sides, a regular formation of intercellular bile canaliculi, and cuboidal cell shapes. Coverage of MOH by Kupffer, Ito, and endothelial cells was increasingly confluent within 1 week in culture. Ito cells could maintain their lipid inclusions even in prolonged







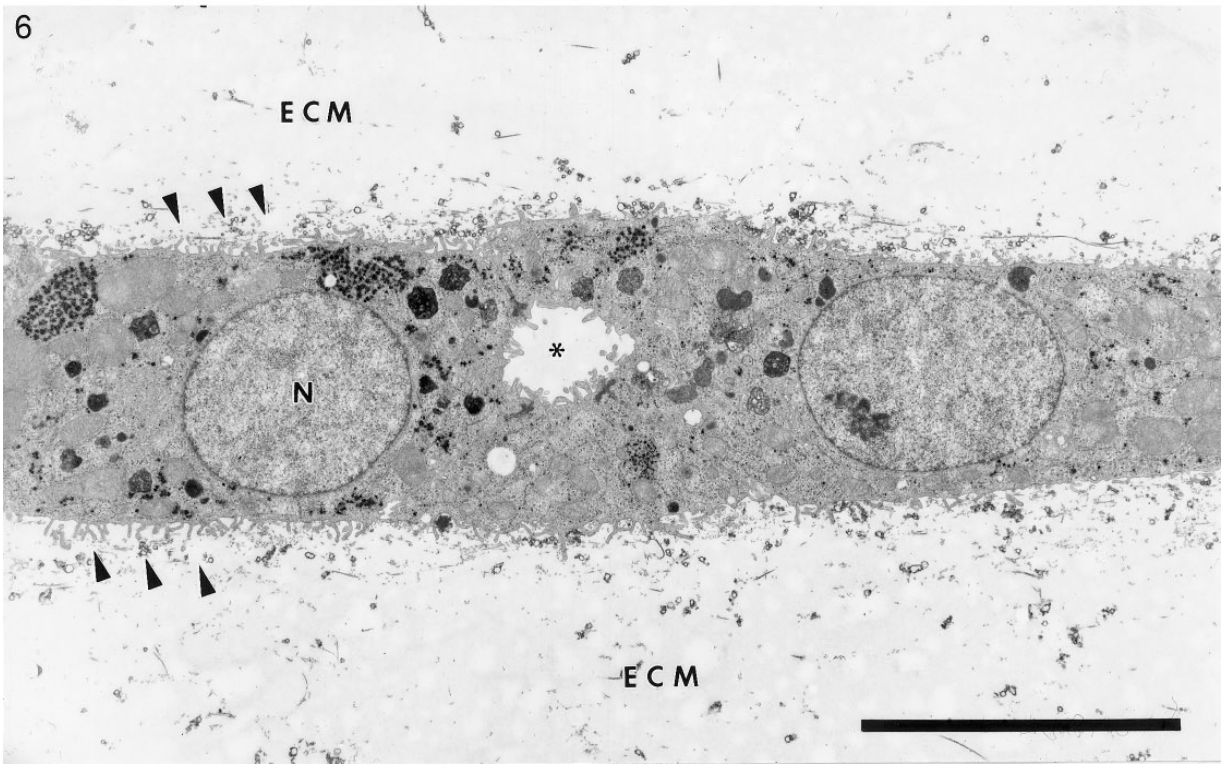


FIG. 4. (A, B) Light microscopic cross-sections of a cell–matrix assembly coculture (NPC indicated by arrowheads) after 2 days (A) and 7 days (B). Cytoplasmic expansions of flat cells cover the superficial collagen layer (ECM). Cuboidal hepatocytes with large round nuclei are located next to each other. At 7 days the superficial NPC layer has become confluent; morphology of its various cell types remains heterogeneous (arrowheads). Bar, 50 μm.

FIG. 5. Overview by TEM of a cross section of the coculture model at Day 1. A Kupffer cell (K), with large cytoplasmatic inclusions is located on top of flat endothelial cells (E), that have an elongated nucleus. Several smaller NPC (arrowheads) are located inside the collagenous matrix (ECM.) Hepatocytes exhibit numerous microprojections on both sinusoidal surfaces and a bile canaliculus (BC). Bar, 10 μm.

culture. PC functions such as albumin secretion as well as phase I and II metabolism of ethoxyresorufin were well maintained. These functions were independent from the presence of NPC. Addition of LPS demonstrated the functional interaction between NPC and hepatocytes by depressing the secretion of albumin.

Model and Morphology

In a previous study [31] we demonstrated that extracellular matrix geometry profoundly modifies the morphological differentiation of primary rat hepatocytes *in vitro.* Hepatocytes overlayed with collagen type I expressed a normal sinusoidal phenotype with numerous microvilli and maintained cell polarity. Direct contact of the hepatocellular sinusoidal surface with culture medium induced the formation of zones free of organelles next to this surface, the loss of cytoplasmic microdifferentiations, and a time-dependent loss of cell polarity. MOH typically had large round nuclei and were higher. The differentiated phenotype with functional polarity of cell organells, cell surface microdifferentiations, expression, and maintenance of intercellular bile canaliculi was also reproducible within the three-dimensional coculture model of this study. Besides extracellular matrix geometry, variations in ECM constituents can influence hepatocytes [32]. ECM within the space of Disse next to the central vein is predominantly composed of collagen type I [33]. The rat tail collagen used in this study also consisted predominantly of collagen type I. Reid *et al.* [34] showed that ECM composed of collagen type I and proteoglycans influenced hepatocyte growth and transcriptory stability of liver specific functions. Naturally occurring matrix components of the liver such as collagen type III [35, 36] may be obtained by coculturing cells of the hepatic sinusoid. In the present study areas underneath NPC with an increased matrix density could be detected.

The notion that form is related to function is reflected also by the functional interaction of PC and NPC *in vivo. In vivo* hepatic nonparenchymal cells occupy a strategic position outside or partially inside the space of Disse enclosing the hepatocytes. Kupffer cells are capable of phagocytosis, antigen processing, secretion of bioactive factors like interleukins upon endotoxin exposure, and cytotoxicity [9]. Endothelial cells equally have defensive functions and act as a sieve in regulating the size of particles, crossing the sinusoidal lining and the space of Disse before reaching the hepatocyte [9]. Fat storing cells are located between the endothelial lining and the hepatocytes [9]. They are involved in the synthesis of ECM including collagens type I, III, IV, fibronectin, laminin, proteoglycans, and a variety of sulfated proteins [37–43]. Standard culture systems completely neglect this structural organization of the hepatic sinusoid. The reestablishment of a more *in vivo*-like three-dimensional configuration is also a time-dependent process in our coculture model. After 1 week in culture extensions of the endothelial cells almost completely covered the superior collagen layer. Kupffer cells were located on top of endothelial cells. This appears to be the result of an active process and resembles the *in vivo* situation of the hepatic sinusoid. Standard cultures of adherent NPC following 48–72 h of incubation resulted in a more than 90% homogeneity of Kupffer cells [44, 45]. In our study the majority of the cocultured NPC after 7 days appeared to be constituted of endothelial cells. In the rat liver *in vivo* [46], 68% of the NPC were identified as endothelial cells. Weibel-Palade bodies are the morphological sites of storage of the von Willebrand factor in peripheral vascular endothelial cells. Whether rat liver endothelial cells, under normal conditions, lack factor VIII-R antigen and Weibel-Palade bodies [47] or not [48] is controversial. However, as fibrosis develops in experimental cirrhosis of rats *in vivo* the morphology and immunohistochemical characteristics of sinusoidal endothelial cells change. Only endothelial cells isolated from fibrotic animals expressed Weibel-Palade bodies [49]. In our study we did not observe Weibel-Palade bodies.

Ito cells were mostly located in between the other NPC and were identified by the presence of lipid droplets [50] even after 1 week in coculture. In myofibroblast transition these cells in standard culture models lose the characteristic lipid droplets [51]. Fat storing cells are known to proliferate in culture [52]. Our system contained insulin and Kupffer cells, factors which have both been shown to stimulate Ito cell proliferation [53]. In our study sections of cytoplasmic processes were detected within the collagen layers and underneath a superficial layer of endothelial and Kupffer cells. Since the NPC were not seeded sequentially and since the collagen layer is prepared free of cells, these NPC could have actively intruded the matrix layer covering the hepatocytes.

In the study we did not detect Pit cells. Pit cells have a lymphoid morphology and can circulate in the blood. *In vivo* they represent 1% of the total NPC population of the liver [54, 55]. These cells were possibly lost during the isolation procedure.

The degree of oxygenation of hepatocytes during the attachment period appears to be critical in determining

FIG. 6. TEM of a cross section of matrix overlaid hepatocytes after 7 days in coculture. Numerous microprojections (arrowheads) are seen on both sinusoidal surfaces. Large roundish nuclei (N), a bile canaliculus (asterisk), many glycogen particles, and intact mitochondria are shown. Bar, 10 μm.

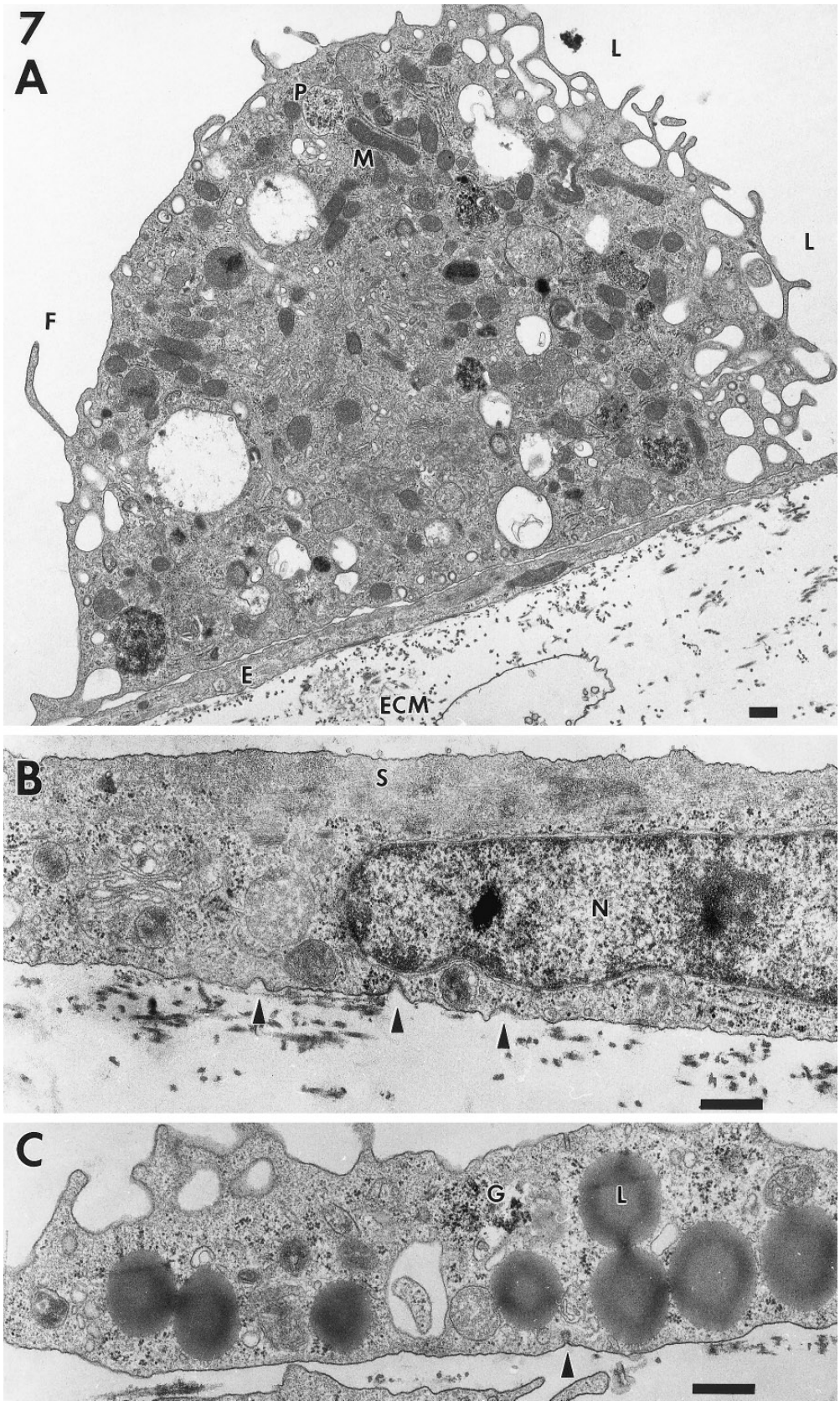


FIG. 7. (A–C) Nonparenchymal cells as seen by TEM at high magnification (bar, 0.5 μm) identifies the roundish cells of Fig. 5 as Kupffer cells (A). Filopodia (F) and lamellopodia (L) are numerously expressed on the surface. Kupffer cells typically show large numbers of phagolysosomes (P) and numerous mitochondria (M). Underneath the Kupffer cell a cytoplasmic extension of an endothelial cell (E) is stretched out on the superior collagen layer (ECM). B shows an endothelial cell with an elongated nucleus (N) and a smooth cell surface. Stress fibers (S) are found on the surface facing the culture medium, whereas the surface toward the ECM is provided with numerous coated pits (arrowheads). Ito cells (C) typically express lipid inclusions (L). Glycogen particles (G) are scattered around the cytoplasm; occasionally coated pits (arrowheads) are found.

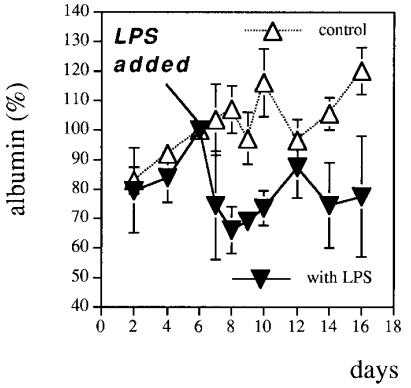


FIG. 8. Effect of LPS stimulation on albumin secretion of MOH in coculture with NPC. Data shown are the average of six measurements of (2×10^6 cells, ELISA).

the attachment efficiency and spreading of the hepatocytes. Using oxygen-impermeable dishes, the best attachment of primary hepatocytes was observed when oxygen concentrations were increased to levels above 160 mmHg [56]. In a three-dimensional coculture model increased cell density due to the superimposed NPCs could reduce the availability of oxygen for the underlaying hepatocytes. Membrane cultures permitted fully confluent hepatocyte layers already at ambient oxygen tension (160 mmHg). The cell density for hepatocytes on a gas-impermeable support in this study corresponded only to 66% if compared to hepatocytes on membranes. Oxygen demand by MOH may, however, be reduced following completion of the attachment and spreading period. During the second week in culture on membranes morphological and biochemical deterioration developed, which could have been caused by oxygen toxicity. Prolonged studies, such as for the acute phase response, therefore needed to be performed using standard petri dishes.

Albumin Secretion and EROD Activity

Interaction between NPC and PC in the coculture model could be demonstrated by a characteristic depression of the negative acute phase protein albumin secretion upon LPS stimulation. In a previous study [21] we showed that a similar behavior can be generated in MOH cultures lacking NPC by the addition of recombinant interleukin-6 (IL-6) to the supernatant. The lowest secretory and transcriptory rates of the negative acute phase protein albumin were equally reached within 24–48 h [21].

Most studies on drug metabolism with hepatocytes have used male rats, which have higher levels of CYP isozymes than females [57]. CYP 1A1 measured as EROD activity was maintained in hepatocytes during cultivation at the same level as Rotem *et al.* [58] have found for female rat hepatocytes in a double-gel cell system. As found also for baseline albumin secretion in our study biotransformation of ethoxyresorufin was not influenced by the presence of NPC. The extracellular matrix geometry of the collagen sandwich configuration appears to be sufficient to stabilize these functions *in vitro.*

MOH have significantly higher cytochrome P450-dependent biotransformation rates of ciclosporin and FK506 than hepatocytes without ECM overlay [23]. This is explained by the stability of cytochrome P450 3A expression and activity in MOH. Human and rat MOH using fibrillar collagen type I from rat tail as in our study metabolized drugs in a species-dependent and *in vivo*-like metabolite pattern [22].

Conclusion

The three-dimensional coculture model between PC and NPC intends to mimic the plate organization of

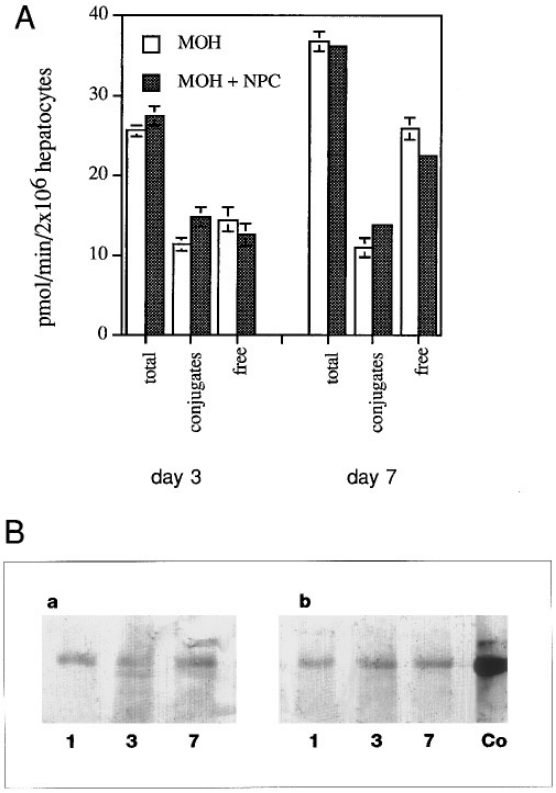


FIG. 9. (A, B) Ethoxyresorufin *O*-deethylase activity and conjugation of resorufin by female rat MOH with and without NPC after 3 and 7 days in culture (A). Both groups maintain stable phase I and II of ethoxyresorufin in culture. Glucuronide and sulfate conjugates of resorufin were cleaved using β-glucuronidase arylsulfatase from *Helix pomatia.* Data is shown as total resorufin (total), free resorufin (free) and amount of conjugates (conjugates). Data points represent the average of three measurements with cultures originating from three different isolations. Western blot (B) of cytochrome P450 1A1 of MOH without (a) and with (b) cocultured NPC. Both groups microsomes.

the liver parenchyma. This model represents a promising and stepwise approach to reconstitute the structure and matrix geometry of the *in vivo* microenvironment of the liver. Observations derived from studies on hepatocytes alone may not fully represent *in vivo* organ function, since the liver has a refined level of tissue assembly and corresponding cellular heterogeneity. Hepatocyte activities are clearly controlled by an organized network of signals transmitted via the matrix and factors derived form NPC *in vivo*. The stepwise reconstruction of liver tissue *in vitro* provides the opportunity to study PC, NPC, and matrix collaboration at different levels of complexity. The effect of oxygen availability in long-term cultures requires further investigation. Ways to define cellular oxygen demand *in vitro* could include studies of cytoprotective enzyme expression as a function of ambient oxygen tension.

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