BIOCHEMICAL PHARMACOLOGY 75 (2008) 1209–1217

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/biochempharm

Cell shape, cell–cell contact, cell–extracellular matrix contact and cell polarity are all required for the maximum induction of CYP2B1 and CYP2B2 gene expression by phenobarbital in adult rat cultured hepatocytes

Hiroaki Oda *, *Yukiko Yoshida*, *Akino Kawamura*, *Atsushi Kakinuma*

Laboratory of Nutritional Biochemistry, Department of Applied Molecular Biosciences, Nagoya University, Nagoya 464-8601, Japan

ARTICLE INFO

Article history:
Received 6 September 2007
Accepted 7 November 2007

Keywords:
Hepatocyte
Phenobarbital
CYP2B
Cell shape
Cell–cell communications
Extracellular matrix
Cell polarity

ABSTRACT

The effect of cell shape, cell density, contact with extracellular matrix and cell polarity on the phenobarbital (PB)-induced gene expression of CYP2B1 and CYP2B2 (CYP2B1/2B2) in adult rat hepatocytes was investigated. High cell density enhanced the induction of CYP2B1/2B2 gene expression by PB. Hepatocytes cultured on EHS gel showed a spherical cell shape and highly enhanced the induction of CYP2B1/2B2 gene expression by PB. Although monolayer hepatocytes cultured on type I collagen (TIC) and type IV collagen exhibited poor induction of CYP2B1/2B2 gene expression by PB, monolayer cells on laminin showed substantial induction. The addition of soluble laminin to media did not show any effect on induction in monolayer hepatocytes cultured on TIC. Dishes coated with different concentrations of immovable laminin demonstrated complicated effects. Coating with higher concentrations of laminin resulted in greater induction of CYP2B1/2B2 gene expression by PB. On the other hand, when hepatocytes were cultured on dishes coated with lower concentrations of laminin, they became round and greater induction of CYP2B1/2B2 gene expression by PB was observed. Spherical hepatocytes cultured on low concentrations of TIC also showed highly enhanced induction of CYP2B1/2B2 gene expression by PB. EHS gel overlay to hepatocytes cultured on TIC and collagen sandwich configurations that are known to induce cell polarity enhanced the induction by PB. The induction of CYP2B1/2B2 gene expression needed cytoskeleton organization, such as actin filament, microtubule filament and intermediate filament. These results demonstrate that cell shape, cell density, contact with extracellular matrix and cell polarity all play critical roles in the induction of CYP2B1/2B2 gene expression by PB.

1. Introduction

Cytochrome P-450 (CYP) comprises a large family of hemoproteins and plays a vital role in the biotransformation of steroids, fatty acids and various kinds of xenobiotics. Some CYPs are highly inducible. Phenobarbital (PB) increases the CYP2B1/2B2 mRNA level in the rat liver [1]. This increase is due to increased transcription of the corresponding CYP2B1 gene

* *Corresponding author*. Tel.: +81 52 789 4124; fax: +81 52 789 5050.
E-mail address: hirooda@agr.nagoya-u.ac.jp (H. Oda).

Abbreviations: apo, apolipoprotein; CAR, constitutive activator receptor; CYP, cytochrome P-450; EHS, Engelbreth-Holm-Swan; IDPN, 3,3′-iminodipropionitrile; PB, phenobarbital; TIC, type I collagen; TIVC, type IV collagen; UGT, UDP-glucuronosyltransferase.

0006-2952/$ – see front matter © 2007 Published by Elsevier Inc.
doi:10.1016/j.bcp.2007.11.003

[2]. Transcriptional activation is rapid and the rate reaches a level 20–50-fold higher than that of the basal transcription rate [3]. CAR (constitutive activator receptor) is thought to be responsible for the PB-dependent induction of CYP2B1/2B2 [4,5]; however, detailed mechanisms of the activation of CAR by PB are still not well understood.

In order to elucidate the precise mechanism of PB-dependent induction of CYP2B1/2B2, the establishment of a well-inducible hepatocyte culture system is required. At present, no established cell lines that respond well to PB treatment exist, although it has been reported that endogenous CYP2B1/2B2 can be induced in primary rat hepatocytes [6]. Various efforts have been made so far to observe sufficient induction of CYP2B1/2B2 in cultured hepatocytes [3,6–9], and the most successful methods have revolved around the manipulation of extracellular matrices. Several studies have established a method whereby primary hepatocytes can be reproducibly cultured on EHS gel (a reconstituted extracellular basement membrane gel from mouse Engelbreth-Holm-Swarm sarcoma) [6–12]. EHS gel is comprised of laminin, type IV collagen (TIVC), proteoglycan, and so on, and permits cells to be spherical. Hepatocytes cultured on dishes coated with EHS gel respond to PB through transcriptional activation of CYP2B1/2B2 genes [6]; however, it remains unclear why hepatocytes cultured on EHS gel can respond to PB and induce CYP2B1/2B2 gene expression.

In the liver, parenchymal hepatocytes contact each other and communicate through gap junctions. Unlike other epithelia, no substantial basement membranes are found close to the basal surfaces of hepatocytes [13]; however, in Disse's space, extracellular matrices such as TIVC, laminin, fibronectin and heparan sulfate proteoglycan, and hepatocytes communicate with extracellular matrices [14]. The hepatocytes are cuboidal and highly polarized [15]. The polarity of hepatocytes is dependent on the maintenance of two distinct membrane domains, apical and basolateral domains. These domains are divided by tight junctions. Normal functions of hepatocytes are considered to be dependent on the polarity of hepatocytes.

In the present study, we investigated the effects of cell shape, cell density, components of extracellular matrix and cell polarity on the induction of CYP2B1/2B2 gene expression in hepatocytes cultured on manipulated extracellular matrices. We demonstrate that all four factors are necessary for the induction of CYP2B1/2B2 gene expression.

2. Materials and methods

2.1. Preparation of hepatocytes

Adult male Wistar rats (150–200 g) were purchased from Japan SLC (Hamamatsu, Japan) and maintained with free access to animal chow and water for 3–10 days prior to use. Rat parenchymal hepatocytes were isolated by perfusing liver with collagenase as described previously [16]. The cells were incubated at an initial density of $1.7 \times 10^5\,cm^{-2}$ (dense) or $4.25 \times 10^4\,cm^{-2}$ (sparse) on ϕ100 mm or ϕ60 mm culture dishes coated with TIC, EHS gel, TIVC or laminin as described below, and hepatocytes were incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. The cultures were maintained in serum- and hormone-free Waymouth's MB 752/1 medium containing penicillin (5 IU/mL) and streptomycin (5 mg/mL). After 4 h, the cells were washed twice with PBS and replaced with fresh warm medium; thereafter, the medium was replaced daily with fresh warm medium.

2.2. Preparation of culture dishes

TIC-coated dishes were prepared by adding different concentrations of TIC (Nitta Gelatin, Japan) (100 μg/mL and 0.5 μg/mL) to plastic dishes (Falcon 1029). EHS gel-coated dishes were prepared as described previously [10]. TIVC-coated dishes were purchased from Collaborative Biomedical Products (USA). Laminin-coated dishes were prepared by adding different concentrations of laminin (Collaborative Biomedical Products, USA) (100 μg/mL, 10 μg/mL, 0.5 μg/mL and 0.1 μg/mL) to plastic dishes (Falcon 1029). TIC gel-coated dishes were prepared as described by Suzuki et al. [17].

2.3. Treatment of hepatocytes

In most experiments, freshly isolated hepatocytes were incubated for 48 h on dishes coated with various substrata. Following incubation, cells were divided and treated with PB (2×10^{-3} M) and with or without dexamethasone (Dex; 1×10^{-6} M) for 24 h. In studies to see the effect of cytoskeleton-disrupting reagents, the cells were cultured for 48 h. Hepatocytes were treated with PB in the presence or absence of cytoskeletal inhibitors, colchicine (1×10^{-4} M), cytochalasin B (1×10^{-5} M), 3,3′-iminodipropionitrile (IDPN, 1%) and okadaic acid (1×10^{-7} M). Colchicine and cytochalasin B were added during the last 24 h of culture, but as okadaic acid showed cytotoxicity, the cells were treated with the drug during the last 4 h. In experiments to add soluble laminin to media, the cells were first plated and cultured on TIC-coated dishes (IWAKI, Japan) for 48 h, then soluble laminin (Collaborative Biomedical Products, USA) was added at a final concentration of 125 μg/mL, and cells were cultured for a further 24 h. In experiments to see the effect of EHS gel overlay, the cells were first plated on TIC-coated dishes (IWAKI, Tokyo, Japan), cultured for 4 h or 48 h, and varying concentrations (20 μg/mL, 100 μg/mL, 200 μg/mL and 500 μg/mL) of EHS gel were added to culture media. In experiments using a TIC gel sandwich, the cells were first plated and cultured on a single layer of TIC gel for 4 h or 48 h, and then a second layer of TIC gel was spread over the cells as described previously [17].

2.4. RNA isolation and Northern blot analysis

Total RNA was extracted according to the method of Chomczyski and Sacchi [18], and RNA was subjected to Northern blot analysis. cDNA clones of rat CYP2B1 [19], rat UDP-glucuronosyltransferase 2 (UGT2) [20] and mouse apolipoprotein E (apo E) [21] were labeled with [5′ α-32P] dCTP using Megaprime DNA Labeling System (Amersham, USA) and used for hybridization. Hybridized bands were visualized autoradiographically. Specific hybridization was quantified with BAS 2000 II (Fuji Film, Japan). The apo E mRNA level was not affected by any treatment, so it was used as the normalization standard.

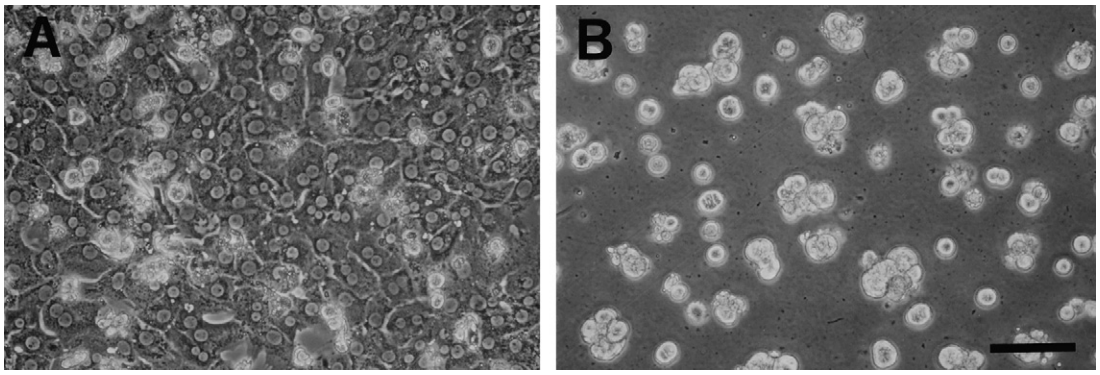

Fig. 1 – Morphologic appearance of rat hepatocytes cultured on plastic dishes coated with type I collagen (A) and EHS gel (B). Hepatocytes were cultured at an initial density of 1.7×10^5 cm^{-2} on plastic dishes coated with TIC and EHS gel in serum- and hormone-free medium for 48 h. Scale bars indicate 50 μm.

2.5. *Western blot analysis*

Microsomal fractions isolated by ultracentrifugation were subjected to Western blot analysis. The filters were treated with goat anti-rat CYP2B1 antibody (Daiichi Kagaku, Japan) and rabbit anti-goat IgG antibody conjugated with horseradish peroxidase (E-Y Laboratories, USA). Immunoreactive proteins were detected using the ECL detection system (Amersham,

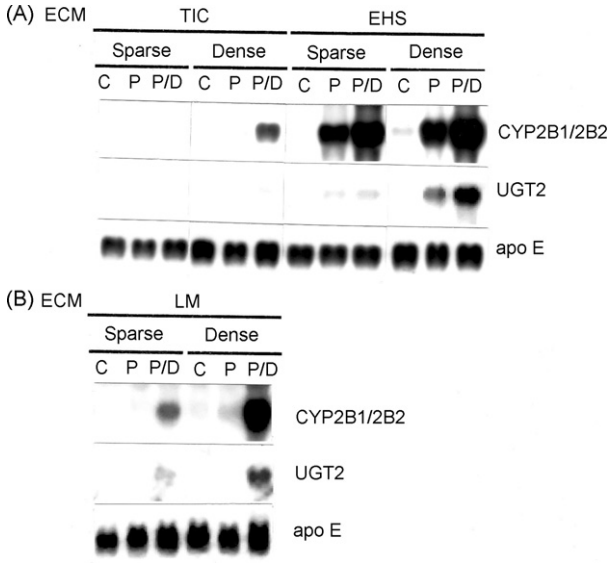

Fig. 2 – Effect of cell density on the induction of CYP2B1/2B2 gene expression by PB in rat hepatocytes cultured on TIC, EHS gel (EHS) and laminin (LM). Hepatocytes were cultured at an initial density of 1.7×10^5 cm^{-2} (dense) or 4.25×10^4 cm^{-2} (sparse) in serum- and hormone-free control medium on plastic dishes coated with TIC, EHS gel (A) or laminin (B) for 48 h. The cells were then incubated in control medium (C), in medium containing PB (2×10^{-3} M) (P), or in medium containing PB (2×10^{-3} M) and Dex (1×10^{-6} M) (P/D) for a further 24 h. Total cellular RNA was extracted and 10 μg of RNA was used for Northern blot analysis. Autoradiogram of Northern blot analysis of CYP2B1/2B2, UGT2 and apo E mRNAs is shown.

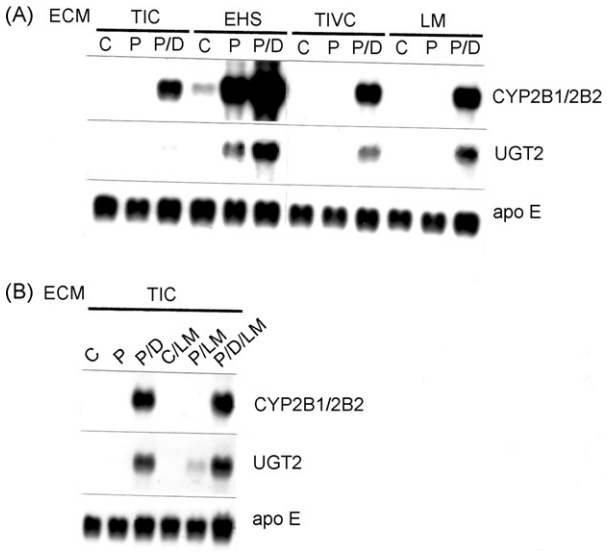

Fig. 3 – Effect of type IV collagen and laminin on the induction of CYP2B1/2B2 gene expression by PB in rat hepatocytes. (A) Hepatocytes were cultured at an initial density of 1.7×10^5 cm^{-2} on plastic dishes coated with TIC, EHS gel (EHS), type IV collagen (TIVC) or laminin (LM) for 48 h. The cells were then incubated in control medium (C), in medium containing PB (2×10^{-3} M) (P), or in medium containing PB (2×10^{-3} M) and Dex (1×10^{-6} M) (P/D) for a further 24 h. (B) Hepatocytes were cultured at an initial density of 1.7×10^5 cm^{-2} on plastic dishes coated with TIC for 48 h. The cells were then incubated in control medium (C), in medium containing PB (2×10^{-3} M) (P), or in medium containing PB (2×10^{-3} M) and Dex (1×10^{-6} M) (P/D), either in the absence or presence of soluble laminin (125 μg/mL) (C/LM, P/LM, P/D/LM) for a further 24 h. Total cellular RNA was extracted and 10 μg of RNA was used for Northern blot analysis. Autoradiogram of Northern blot analysis of CYP2B1/2B2, UGT2 and apo E mRNAs is shown.

USA). Microsomal protein was measured using the Bio-Rad protein assay kit (Bio-Rad, USA).

3. Results

When freshly isolated adult rat hepatocytes were cultured on dishes coated with TIC, they spread and formed a confluent monolayer (Fig. 1A). In contrast, hepatocytes cultured on dishes coated with EHS gel retained a spherical cell shape (Fig. 1B). As we reported previously [7,9], hepatocytes on EHS gel showed high responsiveness to PB (Fig. 2A), whereas in hepatocytes on TIC, the induction of CYP2B1/2B2 gene expression by PB alone was not observed (Fig. 2A) [9]. As shown in Fig. 2, hepatocytes cultured at high density (dense, 1.7×10^5 cells cm^{-2}) on TIC, EHS gel and laminin maintained higher responsiveness to PB than those cultured at low density (sparse, 4.25×10^4 cells cm^{-2}). When Dex was added to medium together with PB, the induction on TIC was observed only in dense culture. The pattern of induction of the UGT2 gene, which is known to encode a phase 2 drug-metabolizing enzyme and to be induced by PB, was similar to that of the CYP2B1/2B2 gene (Fig. 2). These results suggest that cell–cell contact is an important factor for the induction of CYP2B1/2B2 gene expression. When hepatocytes were cultured at low cell density, the induction of CYP2B1/2B2 gene expression was seen in cells on EHS gel or laminin but not on TIC. This indicates that extracellular matrices are important for the induction of CYP2B1/2B2 gene expression.

We next examined the significance of the components of EHS gel, such as TIVC and laminin for the induction of CYP2B1/2B2 gene expression. Hepatocytes cultured on TIVC and laminin formed monolayers similar to those on TIC (data not shown). The level of CYP2B1/2B2 mRNA in the cells on TIVC was almost same as that on TIC, when hepatocytes were treated with both PB and Dex (Fig. 3A). In contrast, the level of CYP2B1/2B2 mRNA in the cells on laminin was higher than that on TIC. This demonstrates that laminin is an important extracellular matrix for the induction of CYP2B1/2B2 gene expression by PB (Fig. 3A) [22]. To determine the effect of laminin on the induction of the CYP2B1/2B2 gene, soluble laminin was added to media. The addition of soluble laminin did not affect either cell morphology (data not shown) or the level of CYP2B1/2B2 mRNA (Fig. 3B), while the induction of UGT2 gene expression by PB was enhanced. Our results suggest that immovable laminin on a dish, but not soluble laminin, enhances the induction of CYP2B1/2B2 gene expression.

We further investigated the significance of laminin in CYP2B1/2B2 gene expression by culturing hepatocytes on varying concentrations of immovable laminin (Fig. 4A–D). As shown in Fig. 5A, hepatocytes cultured on dishes coated with 100 μg/mL of laminin enhanced the induction of CYP2B1/2B2 gene expression by both PB and Dex, whereas induction was not observed in hepatocytes cultured on dishes coated with 10 μg/mL of laminin. Thus, coating with higher concentrations of laminin had an enhancing effect on the induction of CYP2B1/2B2 gene expression; however, cells cultured on both concentrations showed a flat and spread morphology (Fig. 4A and B). On the other hand, substantial induction of CYP2B1/2B2 gene expression was observed in hepatocytes cultured on dishes coated with lower concentrations of laminin, 0.5 μg/mL and 0.1 μg/mL (Fig. 5A), and those hepatocytes exhibited a spherical shape (Fig. 4C and D). Based on these results, we speculate that not only laminin but also cell shape plays important roles in the induction of CYP2B1/2B2.

In order to investigate the role of cell shape on the induction of CYP2B1/2B2 gene expression, hepatocytes were cultured on different concentrations of TIC instead of laminin. When hepatocytes were cultured on a dish coated with 100 μg/mL of TIC, the cells formed a monolayer and the level of the induction of CYP2B1/2B2 gene expression was lower (Figs. 4E

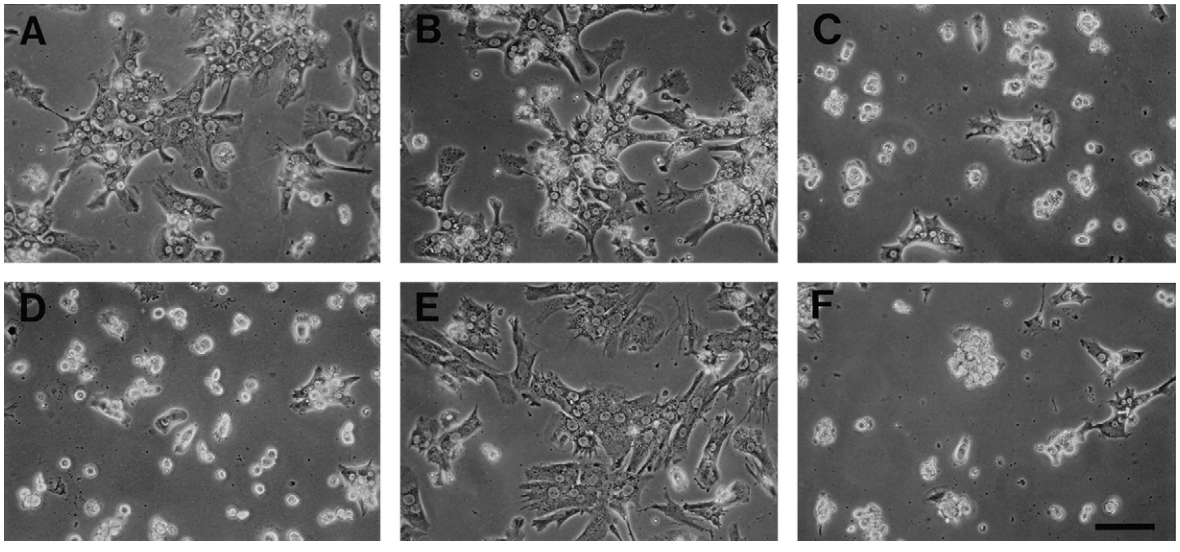

Fig. 4 – Morphologic appearance of rat hepatocytes cultured on plastic dishes coated with immovable laminin and TIC at varying concentrations. Hepatocytes were cultured at an initial density of 4.25×10^4 cm^{-2} for 72 h on plastic dishes coated with 100 μg/mL (A), 10 μg/mL (B), 0.5 μg/mL (C) or 0.1 μg/mL (D) of laminin, or 100 μg/mL (E) or 0.5 μg/mL (F) of TIC. Scale bars indicate 50 μm.

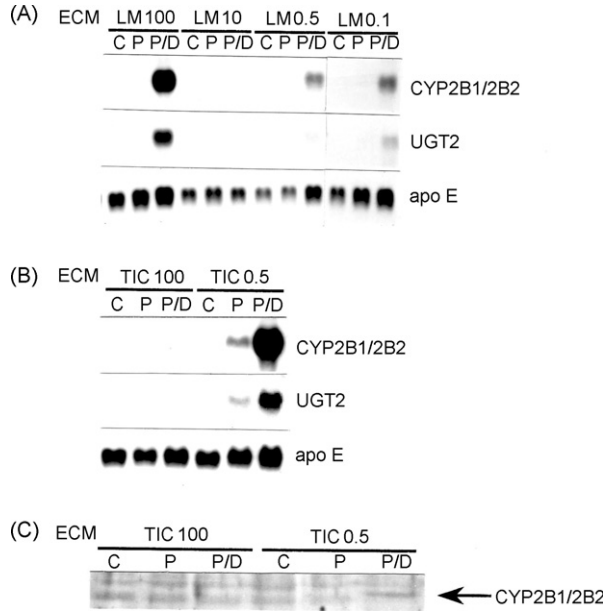

Fig. 5 – Effect of varying concentrations of immovable laminin and TIC on the induction of CYP2B1/2B2 gene expression by PB in rat hepatocytes. (A) Hepatocytes were cultured at an initial density of 4.25×10^4 cm^{-2} on plastic dishes coated with 100 μg/mL (LM 100), 10 μg/mL (LM 10), 0.5 μg/mL (LM 0.5) or 0.1 μg/mL (LM 0.1) of laminin for 48 h, and then the cells were incubated in control medium (C), in medium containing PB (2×10^{-3} M) (P), or in medium containing PB (2×10^{-3} M) and Dex (1×10^{-6} M) (P/D) for a further 24 h. Total cellular RNA was extracted and 10 μg of RNA was used for Northern blot analysis. Autoradiogram of Northern blot analysis of CYP2B1/2B2, UGT2 and apo E mRNAs is shown. (B) Hepatocytes were cultured at an initial density of 4.25×10^4 cm^{-2} on plastic dishes coated with 100 μg/mL (TIC 100) or 0.5 μg/mL (TIC 0.5) of TIC for 48 h, and then the cells were incubated in control medium (C), in medium containing PB (2×10^{-3} M) (P), or in medium containing PB (2×10^{-3} M) and Dex (1×10^{-6} M) (P/D) for a further 24 h. Total cellular RNA was extracted and 10 μg of RNA was used for Northern blot analysis. Autoradiogram of Northern blot analysis of CYP2B1/2B2, UGT2 and apo E mRNAs is shown. (C) Hepatocytes were cultured at an initial density of 4.25×10^4 cm^{-2} on plastic dishes coated with 100 μg/mL (TIC 100) or 0.5 μg/mL (TIC 0.5) of TIC for 48 h, and then the cells were incubated in control medium (C), in medium containing PB (2×10^{-3} M) (P), or in medium containing PB (2×10^{-3} M) and Dex (1×10^{-6} M) (P/D) for a further 24 h. Microsomal fractions were isolated and subjected to Western blot analysis with anti-rat CYP2B1.

and 5B); however, on a dish coated with 0.5 μg/mL of TIC, hepatocytes exhibited a spherical shape and remarkable induction of the genes (Figs. 4F and 5B). Cell shape-dependent induction by PB was observed also at the protein level (Fig. 5C). These results demonstrate that cell shape itself plays a significant role in the induction of CYP2B1/2B2 gene expression. The pattern of induction of the UGT2 gene was similar to that of the CYP2B1/2B2 gene, suggesting that the signal transduction pathway of PB was partly affected by cell shape.

In the liver, hepatocytes are highly organized and maintain cell polarity. We next examined the effect of cell polarity on the induction of CYP2B1/2B2 gene expression. To restore the polarity of cultured hepatocytes, hepatocytes cultured on TIC were overlaid with EHS gel [22], or hepatocytes were sandwiched with TIC gel. Whichever matrix was overlaid, incubation for 24 h did not allow the cells to change their shape (Fig. 6B and E). When hepatocytes were overlaid with EHS gel or TIC gel for 68 h, the cells did not spread well and their shape became irregular (Fig. 6C and F). Both EHS gel overlay and TIC gel sandwich allowed the cells to show substantial induction of CYP2B1/2B2 gene expression by PB (Fig. 7A). In the case of EHS gel overlay, incubation for 24 h was enough for induction (Fig. 7A). These results suggested that the restoration of hepatocyte polarity enhanced the induction of CYP2B1/2B2 gene expression by PB. Maximum induction of CYP2B1/2B2 gene expression was observed in hepatocytes overlaid with 100 μg EHS gel protein/mL (Fig. 7B). The induction of UGT2 gene expression by PB was enhanced by EHS gel overlay, but not by TIC gel sandwich (Fig. 7A), leading us to speculate that cell polarity did not play important roles in the induction of UGT2 gene expression. Since the induction of UGT2 gene expression seemed to be more dependent on laminin than on other extracellular matrices (Fig. 3), the induction of UGT2 gene expression by EHS gel overlay was regarded to be due to laminin contained in EHS gel.

We here demonstrated that cell density, the presence of extracellular matrix, cell shape and cell polarity all played important roles in the induction of CYP2B1/2B2 gene expression by PB. These four factors are all related to cytoskeleton organization directly or indirectly. Change in cell shape and establishment of cell polarity seem to follow the change of cytoskeleton organization. In accordance with the previous finding by Brown et al. [22], colchicine and cytochalasin B inhibited the induction of CYP2B1/2B2 gene expression by PB (Fig. 8). Okadaic acid, a well-known inhibitor of Ser/Thr protein phosphatase, PP1 and PP2A, was reported to act as an inhibitor of intermediate filamentary polymerization [24]. As shown by us [25] and Sidhu and Omiecinski [26], okadaic acid inhibited the induction of CYP2B1/2B2 gene expression by PB (data not shown). An intermediate filament-disrupting reagent, IDPN [27], also inhibited this induction (Fig. 8). These results together led us to conclude that the three cytoskeletons were all crucial factors for the induction of CYP2B1/2B2 gene expression by PB.

4. Discussion

The liver consists of hepatic lobules, which in turn consist of parenchymal cells and nonparenchymal cells. Hepatic parenchymal cells are cuboidal epithelia, and contact each other. Hepatocytes have three distinct membrane domains, basal, lateral and apical domains, and maintain cell polarity. The functions of hepatocytes seem to be affected by extracellular matrices such as collagen, laminin, fibronectin and proteoglycan in Disse's space; however, monolayer hepatocytes cultured under the usual conditions rapidly lose both their cell

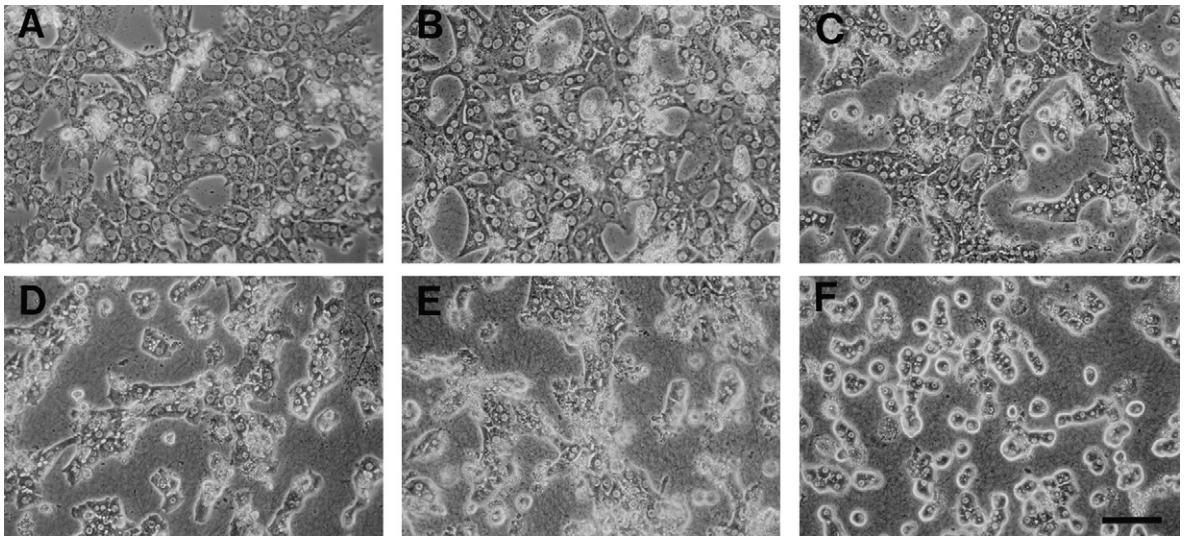

Fig. 6 – Morphologic appearance of rat hepatocytes cultured on plastic dishes coated with various substrata. (A–C) Hepatocytes were cultured at an initial density of 1.7×10^5 cm^{-2} on TIC. The cells were then cultured in medium containing EHS gel (100 μg/mL) for the last 24 h (B) or 68 h (C). For hepatocytes of C, medium containing EHS gel at the same concentration was changed daily. (D–F) Hepatocytes were cultured at an initial density of 1.7×10^5 cm^{-2} on TIC gel (D). The cells were then overlaid with TIC gel for the last 24 h (E) or 68 h (F). All hepatocytes (A–F) were cultured for 72 h. Scale bars indicate 50 μm.

polarity and some liver-specific functions such as the induction of CYP2B1/2B2 by PB. It has been reported that the induction of CYP2B1/2B2 by PB and expression of other liver-specific genes are maintained at a higher level in hepatocytes cultured on EHS gel than in those cultured on TIC [9,10]. There are some apparent differences between hepatocytes on EHS gel and those on TIC, and hepatocytes on EHS gel are thought to be closer to those in the liver. First, EHS gel contains several extracellular matrices such as TIVC, laminin and proteoglycan, which are found in Disse's space. Second, hepatocytes on TIC spread and form a monolayer, while hepatocytes on EHS gel show a spherical cell shape like hepatocytes in the liver. In this study, we investigated the effects of cell shape, cell–cell contact, the presence of extracellular matrix, and cell polarity on the induction of CYP2B1/2B2 gene expression by PB in cultured hepatocytes.

Monolayer hepatocytes on TIVC exhibited poor induction of CYP2B1/2B2 gene expression by PB, like those on TIC (Fig. 3A). On the other hand, hepatocytes on laminin showed more induction of CYP2B1/2B2 gene expression than those on TIC and TIVC (Figs. 2 and 3). It was described previously that hepatocytes on laminin maintained high levels of liver-specific genes expression [22,23]. Brown et al. [22] showed that laminin was important for the induction of CYP2B1/2B2 gene expression. They also indicated that soluble laminin and synthetic peptides of laminin fragments effectively enhanced induction; however, we found that the addition of laminin to media did not affect CYP2B1/2B2 gene expression (Fig. 3B). Although the conflicting results are puzzling, both our results and those of Brown et al. [22] indicate that at least immovable laminin enhances the induction of CYP2B1/2B2 gene expression by PB.

As shown in Fig. 5A, the effect of laminin on CYP2B1/2B2 gene expression was slightly complicated, and the concentration versus efficacy curve showed a U-shaped pattern. Namely, hepatocytes cultured on dishes coated with larger or smaller amounts of laminin showed the induction of CYP2B1/2B2 gene expression by PB, whereas hepatocytes on dishes coated with an intermediate amount of laminin did not (Fig. 5A). We speculated that the U-shaped action of laminin could be integrated by two factors, laminin itself and the cell shape. When hepatocytes exhibit a spread morphology, the quantity of laminin coated on dishes is important for induction. In other words, more laminin allows hepatocytes more induction. When hepatocytes show a spherical morphology on dishes with a limited amount of laminin, the induction of CYP2B1/2B2 gene expression by PB is enhanced. To assess the role of cell shape, we tried to change the morphology of hepatocytes by TIC instead of laminin. Spherical hepatocytes on dishes coated with a smaller amount of TIC exhibited a remarkable induction of CYP2B1/2B2 gene expression (Fig. 5B). It was reported previously that hepatocytes on dishes coated with a limited amount of collagen, laminin or fibronectin exhibited a spherical morphology and maintained liver phenotype [28]. These results indicated that the spherical shape of hepatocytes was necessary for the induction of CYP2B1/2B2 gene expression.

Caron [23] reported that when dilute EHS gel was added to culture medium following cell attachment to a conventional substratum of TIC (EHS gel overlay), transcription of the albumin gene was maintained at a higher level. As shown in Fig. 7, EHS gel overlay caused a robust induction of CYP2B1/2B2 gene expression by PB. These results are consistent with those of previous reports [29,30]. Musat et al. [31] reported that EHS gel overlay enabled the reestablishment of cell polarity by

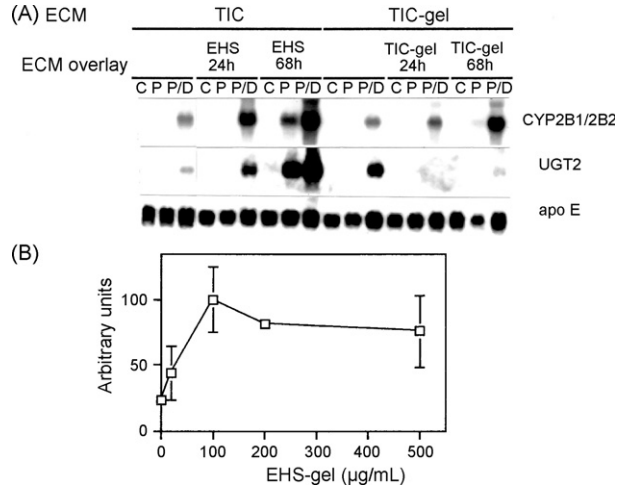

Fig. 7 – Effect of EHS gel overlay and TIC gel sandwich on the induction of CYP2B1/2B2 gene expression by PB. (A) Hepatocytes were cultured at an initial density of 1.7×10^5 cm^{-2} on TIC or TIC gel. The cells were then overlaid with EHS gel (100 μg/mL) or TIC gel for the last 24 h or 68 h. Hepatocytes were treated with PB (2×10^{-3} M) (P), or PB (2×10^{-3} M) and Dex (1×10^{-6} M) (P/D) for the last 24 h. Total cellular RNA was extracted and 10 μg of RNA was used for Northern blot analysis. Autoradiogram of Northern blot analysis of CYP2B1/2B2, UGT2 and apo E mRNAs is shown. (B) Hepatocytes were cultured at an initial density of 1.7×10^5 cm^{-2} on TIC for 48 h. The cells were then cultured in medium containing EHS gel (500 μg/mL, 200 μg/mL, 100 μg/mL and 20 μg/mL) plus PB (2×10^{-3} M) and Dex (1×10^{-6} M) for a further 24 h. Total cellular RNA was extracted and 10 μg of RNA was used for Northern blot analysis. Quantitative analysis of specific hybridization was performed with BAS 2000 II. Values of CYP2B1/2B2 mRNA were normalized with those of apo E mRNA. CYP2B1/2B2 mRNA levels are expressed as a percentage relative to those of hepatocytes cultured in medium containing 100 μg/mL EHS gel. Each value and vertical bar is expressed as the mean and S.E.M. of three independent experiments, respectively.

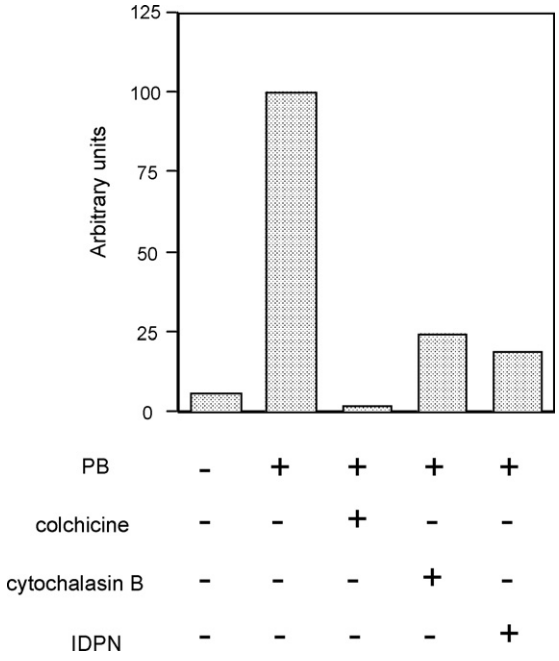

Fig. 8 – Effect of cytoskeleton-disrupting reagents on the induction of CYP2B1/2B2 gene expression by PB in rat hepatocytes cultured on EHS gel. Hepatocytes were cultured at an initial density of 1.7×10^5 cm^{-2} on plastic dishes coated with EHS gel in a serum- and hormone-free medium for 24 h, and cells were treated with PB (2×10^{-3} M) and colchicine (1×10^{-4} M), cytochalasin B (1×10^{-5} M) and IDPN (1%) for a further 24 h. Fifteen micrograms of total RNA was used for Northern blot analysis. Quantitative analysis of specific hybridization was performed with BAS 2000 II (Fuji Film). Values of CYP2B1/2B2 mRNA were normalized with those of apo E mRNA. CYP2B1/2B2 mRNA levels are expressed as a percentage relative to the values of hepatocytes treated with PB alone. Each value is expressed as the mean of two independent experiments.

demonstrating various intracellular events, including the reformation of γ-glutamyltransferase, Mg^{2+}-ATPase-positive bile canalicular networks, localization of gap-junction proteins to the lateral membrane, reorganization of the actin cytoskeleton into pericanalicular webs and location of Golgi complexes in mitochondria-poor pericanalicular cytoplasm. These results suggested that enhancement of the induction of CYP2B1/2B2 gene expression by EHS gel overlay was caused by the reestablishment of cell polarity of hepatocytes. Cell polarity of hepatocytes was also reestablished by a collagen sandwich system to avoid the possibility that laminin itself contained in EHS gel enhanced induction in the EHS gel overlay system. It has been reported that the collagen sandwich system causes a reorganization of cytoskeleton, adoption of *in vivo*-like morphology and polarity, and expression of liver-specific functions [17,32–36]. Hepatocytes cultured in a TIC gel sandwich system also responded to PB with the induction of CYP2B1/2B2 gene expression (Fig. 7). This demonstrated that the cell polarity of hepatocytes played an important role in the induction of CYP2B1/2B2 gene expression by PB. Because the TIC gel sandwich for 24 h did not seem to be sufficient to reestablish cell polarity [15], hepatocytes cultured in a TIC gel sandwich system showed a similar level of CYP2B1/2B2 mRNA to those on a single layer of TIC gel. In contrast, EHS gel overlay for 24 h enhanced the induction of CYP2B1/2B2 gene expression substantially. The difference between EHS gel overlay and TIC gel sandwich depends on various components of EHS gel, such as laminin. On the other hand, when either EHS gel or TIC gel was overlaid for 68 h, hepatocytes showed an irregular-shaped morphology (Fig. 6). As shown in Fig. 5, cell shape played a critical role in the induction of CYP2B1/2B2 gene expression; therefore, enhancement of induction by both EHS gel and TIC gel overlay is due to not only cell polarity but also cell shape. We are now devising a method which can segregate the effects of these two factors.

The ability of cells to adopt a variety of shapes and polarity depends on a complex network of protein filaments called the

cytoskeleton, which consists of three kinds of cytoskeleton filaments, actin filaments, microtubules and intermediate filaments. We confirmed that PB-induced expression of the CYP2B1/2B2 gene and UGT2 gene was inhibited by colchicine, cytochalasin B and IDPN (Fig. 8). These results indicated that the cytoskeleton organization was essential for the PB-signaling pathway.

In conclusion, the results from this study demonstrate that cell shape, cell–cell contact, cell–extracellular matrix contact and cell polarity are all important for the induction of CYP2B1/2B2 gene expression by PB. Our results suggest that the difficulties to maintain the responsiveness to PB in cultured hepatocytes come from the requirement of hepatocytes for all four factors in culture.

Acknowledgments

We thank Drs. Y. Fujii-Kuriyama, S. Tajima and F. Horio for the generous gifts of cDNAs. We also thank Dr. K. Kimata for the kind gift of EHS sarcoma.

REFERENCES

[1] Whitlock JP. The regulation of cytochrome P-450 gene expression. Annu Rev Pharmacol Toxicol 1986;26:333–69.
[2] Hardwick JP, Gonzalez FJ, Kasper CB. Transcriptional regulation of rat liver epoxide hydratase, NADPH-Cytochrome P-450 oxidoreductase, and cytochrome P-450b genes by phenobarbital. J Biol Chem 1983;258:8081–5.
[3] Waxman DJ, Azaroff L. Phenobarbital induction of cytochrome P-450 gene expression. Biochem J 1992;281: 577–92.
[4] Kawamoto T, Sueyoshi T, Zelko I, Moore R, Washburn K, Negishi M. Phenobarbital-responsive nuclear translocation of the receptor CAR in induction of the CYP2B gene. Mol Cell Biol 1999;19:6318–22.
[5] Wei P, Zhang J, Egan-Hafley M, Liang S, Moore DD. The nuclear receptor CAR mediates specific xenobiotic induction of drug metabolism. Nature 2000;407: 920–3.
[6] Schuetz EG, Li D, Omiecinski CJ, Muller-Eberhard U, Kleinman HK, Elswick B, et al. Regulation of gene expression in adult rat hepatocytes cultured on a basement membrane matrix. J Cell Physiol 1988;134:309–23.
[7] Matsushita N, Oda H, Kobayashi K, Akaike T, Yoshida A. Induction of cytochrome P-450s and expression of liver-specific genes in rat primary hepatocytes cultured on different extracellular matrices. Biosci Biotechnol Biochem 1994;58:1514–6.
[8] Nemoto N, Sakurai J, Funae Y. Maintenance of phenobarbital-inducible Cyp2b gene expression in C57BL/6 mouse hepatocytes in primary culture as spheroids. Arch Biochem Biophys 1995;316:362–9.
[9] Yoshida Y, Kimura N, Oda H, Kakinuma A. Insulin suppresses the induction of CYP2B1 and CYP2B2 gene expression by phenobarbital in adult rat cultured hepatocytes. Biochem Biophys Res Commun 1996;229: 182–8.
[10] Oda H, Nozawa K, Hitomi Y, Kakinuma A. Laminin-rich extracellular matrix maintains high level of hepatocyte nuclear factor 4 in rat hepatocyte culture. Biochem Biophys Res Commun 1995;212:800–5.
[11] Oda H, Nozawa K, Miyachi F, Shimizu A, Iwasaki Y, Kakinuma A. High responsiveness to thyroid hormone of adult rat primary hepatocytes cultured on EHS-gel. Biosci Biotech Biochem 1997;61:1590–2.
[12] Oda H. Extracellular matrix regulates hepatocyte gene expression and differentiation. Connect Tissue 1998;30:225–32.
[13] Hughes RC, Stamatoglou SC. Adhesive interactions and the metabolic activity of hepatocytes. J Cell Sci Suppl 1987;8:273–91.
[14] Stamatoglou SC, Hughes RC. Cell adhesion molecules in liver function and pattern formation. FASEB J 1994;8: 420–7.
[15] Berthiaume F, Moghe PV, Toner M, Yarmush ML. Effect of extracellular matrix topology on cell structure, function, and physiological responsiveness: hepatocytes cultured in a sandwich configuration. FASEB J 1996;10:1471–84.
[16] Nagaoka S, Kamuro H, Oda H, Yoshida A. Effects of polychlorinated biphenyls on cholesterol and ascorbic acid metabolism in primary cultured rat hepatocytes. Biochem Pharmacol 1991;41:1259–61.
[17] Suzuki M, Ichikawa K, Sakoda A, Sakai Y. Long-term culture of primary rat hepatocytes with high albumin secretion using membrane-supported collagen sandwich. Cytotechnology 1993;11:213–8.
[18] Chomczyski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. Anal Biochem 1987;162:156–9.
[19] Fujii-Kuriyama Y, Mizukami Y, Kawajiri K, Sogawa S, Muramatsu M. Primary structure of a cytochrome P-450: coding nucleotide sequence of phenobarbital-inducible cytochrome P-450 cDNA from rat liver. Proc Natl Acad Sci USA 1982;79:2793–7.
[20] Horio F, Naito Y, Hattori S, Yoshida A. A low protein diet increases 4-nitrophenol-UDPglucuronosyltransferase but decreases chloramphenicol-UDPglucuronosyltransferase activity and mRNA level in livers of rats treated with polychlorinated biphenyls. Biosci Biotechnol Biochem 1995;59:1237–41.
[21] Horiuchi K, Tajima S, Nenju M, Yamamoto A. Structure and expression of mouse apolipoprotein E gene. J Biochem 1989;106:98–103.
[22] Brown SES, Guzelian CP, Schuetz E, Quattrochi LC, Kleinman HK, Guzelian PS. Critical role of extracellular matrix on induction by phenobarbital of cytochrome P450 2B1/2 in primary cultures of adult rat hepatocytes. Lab Invest 1995;73:818–27.
[23] Caron JM. Induction of albumin gene transcription in hepatocytes by extracellular matrix proteins. Mol Cell Biol 1990;10:1239–43.
[24] Blankson H, Holen I, Seglen PO. Disruption of the cytokeratin cytoskeleton and inhibition of hepatocytic autophagy by okadaic acid. Exp Cell Res 1995;218:522–30.
[25] Kawamura A, Yoshida Y, Kimura N, Oda H, Kakinuma A. Phosphorylation/dephosphorylation steps are crucial for the induction of CYP2B1 and CYP2B2 gene expression by phenobarbital. Biochem Biophys Res Commun 1999;264:530–6.
[26] Sidhu J, Omiecinski CJ. An okadaic acid-sensitive pathway involved in the phenobarbital-mediated induction of CYP2B gene expression in primary rat hepatocyte cultures. J Pharmacol Exp Ther 1997;282:1122–9.
[27] Galigniana MD, Scruggs JL, Herrington J, Welsh MJ, Carter-Su C, Housley PR, et al. Heat shock protein 90-dependent (geldanamycin-inhibited) movement of the glucocorticoid receptor through the cytoplasm to the nucleus requires intact cytoskeleton. Mol Endocrinol 1998;12:1903–13.
[28] Mooney D, Hansen L, Vacanti J, Langer R, Farmer F, Ingber D. Switching from differentiation to growth in hepatocytes:

control by extracellular matrix. J Cell Physiol 1992;151:497–505.
[29] Sidhu JS, Farin FM, Omiecinski CJ. Influence of extracellular matrix overlay on phenobarbital-mediated induction of CYP2B1, 2B2, and 3A1 genes in primary adult rat hepatocyte culture. Arch Biochem Biophys 1993;301:103–13.
[30] Sidhu JS, Farin FM, Kavanagh TJ, Omiecinski CJ. Effect of tissue-culture substratum and extracellular matrix overlay on liver-selective and xenobiotic inducible gene expression in primary rat hepatocytes. In Vitro Toxicol 1994;7:225–42.
[31] Musat AI, Sattler CA, Sattler GL, Pitot HC. Reestablishment of cell polarity of rat hepatocytes in primary culture. Hepatology 1993;18:198–205.
[32] Dunn JCY, Yarmush ML, Koebe HG, Tompkins RG. Hepatocyte function and extracellular matrix geometry: long-term culture in a sandwich configuration. FASEB J 1989;3:174–7.
[33] Dunn JCY, Tompkins RG, Yarmush ML. Long-term in vitro function of adult hepatocytes in a collagen sandwich configuration. Biotechnol Prog 1991;7:237–45.
[34] Dunn JCY, Tompkins RG, Yarmush ML. Hepatocytes in collagen sandwich: evidence for transcriptional and translational regulation. J Cell Biol 1992;116:1043–53.
[35] Ezzell RM, Toner M, Hendricks K, Dunn JCY, Tompkins RG, Yarmush ML. Effect of collagen gel configuration on the cytoskeleton in cultured rat hepatocytes. Exp Cell Res 1993;208:442–52.
[36] LeCluyse EL, Audus KL, Hochman JH. Formation of extensive canalicular networks by rat hepatocytes cultured in collagen-sandwich configuration. Am J Physiol 1994;266:C1764–7.