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# Effects of epidermal growth factor on CYP inducibility by xenobiotics, DNA replication, and caspase activations in collagen I gel sandwich cultures of rat hepatocytes

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**Abstract**

In this study, we investigated the combined effects of EGF and collagen I gel on the phenotype of cultured rat hepatocytes and we focussed our investigations on the regulation of xenobiotic-mediated induction of CYP, cell cycle progression and activation of capases 8 and 3. We found that EGF, added to basal culture medium or phenobarbital (3.2 mM) containing medium, provoked a moderate decrease of CYP1A1 and CYP2B1/2 activities. However, EGF did not exert any inhibitory effect on 3-methylcholantrene (5 $\mu$M) and $\beta$-naphtoflavone (25 $\mu$M) induction of CYP1A1 activities. In collagen gel sandwich cultures, hepatocytes remained arrested in mid-G1 phase of the cell cycle, even in the presence of EGF. In conventional primary cultures, caspases 8 and 3 were activated at 3 and 5 days after plating respectively. In collagen gel sandwich cultures, we found that neither collagen I nor EGF prevented activation of caspase 8 while collagen I gel inhibited activation of caspase 3, preventing spontaneous apoptosis of cultured rat hepatocytes. In contrast, EGF transiently increased caspase 3 activity at day 1 after plating. Altogether, our data demonstrate that collagen I gel triggers intracellular signals which strongly affect cultured hepatocyte phenotype, leading to a cell cycle arrest in G1 phase and long-term survival through the inhibition of caspase 3 activation and that EGF-free medium improves survival and liver-specific gene expression in hepatocytes maintained in collagen I gel sandwich cultures. 

*Keywords:* CYP1A1; CYP2B; Cell cycle; c-*fos*; c-*jun*; c-*myc*; Cyclin D1; Cdk1; Apoptosis

## 1. Introduction

*In vivo*, adult hepatocytes remain in a quiescent phase (G0) and express differentiated hepatic functions including drug metabolism capacity [1]. They can, in contrast with terminally differentiated cells, re-enter the cell cycle, for example in response to partial hepatectomy or chemical liver injury, and proliferate to allow liver regeneration [2]. This process is regulated by a complex combination of priming and growth factors which control entry into and progression through the G1 phase of the cell cycle [3]. During hepatocyte proliferation, transient changes in the expression of adult liver specific genes occur, including an increase of fetal isoforms of plasma proteins and metabolism enzymes [4] and a decrease in CYP content [5,6].

*In vitro*, the response of adult rat hepatocytes to growth factors has been studied extensively with respect to DNA synthesis and proliferation [7]. Normal adult rat hepatocytes spontaneously transit from G0 to G1 during isolation of the liver. In primary cultures, they do not proliferate without addition of mitogenic factors and are arrested at a mitogen-dependent restriction point in mid-late G1 phase of the cell cycle. In the presence of mitogens, they progress in late G1, enter S-phase and divide [8–10]. In conventional primary culture in which hepatocytes are plated on plastic or thin

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*Abbreviations:* BrdU, 5-bromo 2′-deoxyuridine; Cdk 1, Cyclin dependent kinase 1; CYP, cytochrome P450; DEVD-AMC, N-acetyl-Asp-Glu-Val-Asp-aminomethylcoumarin; EGF, epidermal growth factor; EROD, ethoxyresorufin O-deethylase; 3-MC, 3-methylcholantrene; $\beta$-NF, $\beta$-naphtoflavone; PB, phenobarbital; and PROD, pentoxyresorufin O-dependentylase.

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coatings of collagen I or fibronectin, expression of liver specific functions rapidly decreases and cell survival does not exceed a few days [11] due to a spontaneous apoptosis [12] which involves the activation of several cysteinic aspartic proteases (caspases) including caspases 8 and 3 [13]. These phenotypic changes constitute a limitation in the use of hepatocyte primary cultures for various applications such as pharmacology and toxicology.

There have been many attempts to develop a serum-free primary hepatocyte culture system in which the cells would survive and keep their differentiated state for a long period of time. Extensive improvements have been obtained by changing the culture conditions through the use of: (i) culture medium additives, including hormones, nicotinamide, pyruvate, phenobarbital and dimethylsulfoxide [11], (ii) cocultures associating hepatocytes and non-parenchymal liver epithelial cells [14], (iii) complex extracellular matrices such as Vitrogen® [15], Matrigel® [16] crude liver membrane fractions [17] and collagen type I gel [18].

The use of collagen I gel has led to two long-term survival culture models of hepatocytes, namely the collagen gel sandwich culture [18] and the so-called collagen gel immobilisation culture [19]. In both models, inducible CYP dependent enzymes [20,21,22,23,24], microsomal epoxide hydrase [23] and glutathione S-transferase [25] expressions are maintained to a certain extent demonstrating that hepatocytes are well differentiated in these culture models. To date, there is no report describing the expression and activity of caspases in these collagen I gel cultures although the hepatocyte survival in these models is greatly improved suggesting the inhibition by collagen I gel sandwich of the spontaneous apoptosis occurring in conventional primary cultures [12,13].

In conventional primary culture, EGF induces many effects. It stimulates proliferation, increases cell survival [7] and inhibits transforming growth factor-$\beta$ (TGF$\beta$)-induced apoptosis in hepatocytes [26] but does not significantly improve expression of liver-specific functions. In collagen gel cultures, EGF is traditionally added to the culture medium, in accordance with the authors who described the methodology for the first time [18]. It has been reported that EGF decreases xenobiotic-mediated induction of CYP expression in cultured human [27] and rodent hepatocytes [6,28]. The effects of EGF on proliferation, differentiation and survival of hepatocytes cultured in collagen I gel sandwich have not been fully addressed. However, Hansen and Albrecht [9], have recently demonstrated that hepatocytes cultured on a monolayer of collagen I gel or dried collagen, do not respond to growth factors and remain arrested in G1 phase of the cell cycle. In contrast, hepatocytes plated on a rigid film of collagen I can enter S phase and divide. These results indicate that the mitogenic effect of EGF on hepatocyte cell cycle is dependent upon the type of collagen I coating used to initiate the culture. Experiments performed in our laboratory, have also shown that the omission of EGF from the culture medium results in higher phase I enzymatic activities in hepatocytes maintained in collagen I sandwich [24] demonstrating that EGF is able to modulate CYP expression in differentiated hepatocytes.

The aim of this work was to investigate, in normal adult rat hepatocytes maintained in collagen I gel cultures, the effects of EGF on liver specific gene expression, especially drug-mediated induction of CYP, cell cycle progression and the expression and activation of caspases 3 and 8.

## 2. Methods

### 2.1. Chemicals

Bovine serum albumin (BSA) fraction V, EGF, crude collagenase (type I), ethoxyresorufin, pentoxyresorufin, resorufin, bovine insulin, piperazine-*N*,*N*′-*bis*-(2-ethanesulfonic acid) (PIPES), and 3-[3-cholamidopropyl-dimethylammonio]-2-hydroxy-l-propanesulfonic acid (CHAPS), orthovanadate, benzamide, aprotinin, leupeptin, and soybean trypsin inhibitor were purchased from Sigma Chemical Company. Rediprime II DNA labeling kit, DNA herring sperm, [$\alpha$-$^{32}$P]dCTP (3000 Ci/mmol), [$^{3}$H]-methyl-thymidine (25 Ci/mmol), CYP1A1 ELISA system, CYP2B1/2 ELISA system, and BrdU labeling kit were purchased from Amersham Life Sciences. Dulbecco's Modified Eagles Medium (DMEM) with 4.5 mg/mL glucose and L-glutamine came from B.I. BioWithacker. Cdk1, cyclin D1, c-*jun* c-*fos* and c-*myc* were obtained as described [8]. Rabbit polyclonal anti-caspase-8 (APP-108) was from Stressgen while the secondary antibody conjugated to horseradish peroxidase was purchased from DAKO. Fluorogenic DEVD-AMC substrate was from BACHEM (France). Supersignal™ came from Pierce Chemical Co. All other products were readily available commercial products.

### 2.2. Animals

Male outbred Sprague–Dawley rats (200–300 g) were obtained from IffaCredo. They were kept under controlled environmental conditions (12-hr light-dark cycle) and fed a standard diet (Animalabo A 04, water ad libitum). After surgical removal of 70% of the liver, regenerating liver samples are obtained 24 hr after partial hepatectomy. The livers were harvested, immediately frozen in liquid nitrogen and stored until analysis [29]. Procedures for housing the rats and isolating and culturing rat hepatocytes were approved by the local ethical committee of the Vrije Universiteit Brussel.

### 2.3. Isolation and culture of hepatocytes

Primary rat hepatocytes were isolated as described previously [30]. Cell integrity was tested by trypan blue exclusion and was between 80 and 86%. Hepatocytes were cultured either in collagen type I sandwich and immobilisation

gel culture systems [31] or directly on plastic (Falcon) culture dishes [32] or in Costar 6-well plates (DNA synthesis assay) as described.

### 2.4. Hepatocyte treatment

Rat hepatocytes were cultured in the presence and absence of EGF (0.02 μg/mL) or alternatively EGF was added after 2 days in culture and this for 24 hr. In the induction studies, rat hepatocyte cultures were daily treated with 3.2 mM PB, 25 μM β-NF, and 5 μM 3-MC in the presence and absence EGF (0.02 μg/mL) or when EGF was added after 2 days in culture and this for 24 hours. Inducers were added to the cultures as concentrated stock solutions in water (for PB) or in dimethylsulfoxide (DMSO) for 3-MC and β-NF. A final concentration of 0.05% (v/v) DMSO was present in the culture medium. Treatment started 4 hours after seeding the cells.

### 2.5. Determination of albumin secretion

Medium samples collected at days 2, 4, and 7 were analysed for their albumin content by an enzyme-linked immunosorbent assay (ELISA technique) according to Dunn *et al*. [33].

### 2.6. Determination of CYP-dependent activities

Microsomes were prepared from freshly isolated hepatocytes and hepatocytes cultured for 7 days according to Hales and Neims [34]. The microsomes were incubated with 5 μM ethoxy- or penthoxyresorufin. The resorufin formed was measured fluorimetrically according to Burke and Mayer [35]. Enzymatic activities of EROD and PROD have been expressed versus microsomal protein content.

### 2.7. Determination of microsomal proteins

Microsomal protein concentrations were determined according to the procedure of Bradford [36] using Bio-Rad reagent and with BSA as a standard.

### 2.8. DNA BrdU incorporation

The BrdU incorporated during DNA replication was detected using an indirect immunocytochemistry method. BrdU was diluted in the culture medium (1/1000) and incubation was performed at 37° during 24 hr. The cells were fixed for 30 min in solution of ethanol (90%) and acetic acid (5%), then washed three times with PBS. Nonspecific sites were saturated by PBS supplemented with 10% fetal calf serum, during 20 min. BrdU was detected using a mouse anti-BrdU specific monoclonal antibody. Then, anti-BrdU antibodies were detected using a secondary anti-mouse IgG2A immunoglobulin coupled to Horse-Radish Peroxidase. Positive cells were then revealed with 3-3′diaminobenzidine tetrahydrochlorure.

### 2.9. RNA extraction

Freshly isolated hepatocytes and cultured cells were harvested, washed three times with PBS and stored as pellets at −80°. Total RNA was extracted from cell pellets according to Xie and Rothblum [37] by the thiocyanate-guanidinium procedure. Integrity of the RNA samples was confirmed by formaldehyde agarose gel electrophoresis and visualisation by ethidium bromide staining. The quality of isolated RNA from control and treated hepatocytes was evaluated by the presence of intact 18S and 28S ribosomal RNAs.

### 2.10. Northern blot analysis

Total RNAs were purified using SV Total RNA isolation system kit (Promega) according manufacturer instructions. Total RNAs were loaded on a denaturing 1.2% agarose gel buffered with 10 mM phosphate, pH 7.4 and containing 1.1 M formaldehyde, and then transferred onto hybond $N^+$ filters (Amersham) as described by Thomas [38].

Filters were prehybridized and hybridized with the cDNAs of interest labelled with [$^{32}$P] dCTP (3000 Ci/mmol) (Rediprime™, Amersham) according to Andrews *et al*. [39]. Equal mRNA loading was estimated using 18S ribosomal probe.

### 2.11. Fluorimetric assay of caspases activity

Freshly isolated hepatocytes and cultured cells were harvested and washed three times with PBS and stored as pellets at −80°. After thawing, hepatocytes were lysed in a DEVD-AMC caspase 3-like activity buffer containing 20 mM PIPES pH 7.2, 100 mM NaCl, 10 mM dithiotreitol, 1 mM EDTA, 0.1% CHAPS, 10% sucrose. 100 μg of crude cell lysate was incubated with 100 μM DEVD-AMC caspase 3-like substrate at 37° for 1 hr. Caspase 3-mediated cleavage of DEVD-AMC peptide was measured by spectrofluorometry using excitation/emission wavelength pairs (λex/λem) of 380/440 nm. The caspase activity was presented as fluorescence arbitrary units per 100 μg of total proteins. The background of fluorescence in each reaction after mixture of all constituents was subtracted from the final value. Fluorogenic DEVD-AMC substrate was reconstituted at 100 mM in 1% $NH_4OH$.

### 2.12. Immunoblotting analysis

Freshly isolated hepatocytes and cultured cells were harvested, washed three times with PBS and stored as pellets at −80°. After thawing, hepatocytes were lysed in a Western blot lysis buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 15 mM $MgCl_2$, 1 mM EDTA, 2.5 mM EGTA, 1 mM dithiotreitol, 0.1% Tween 20%, 0.1 mM sodium orthovanadate, 1 mM NaF, 10 mM b-glycerophosphate, 0.1 mM phenylmethylsulfonyl fluoride, 100 mg/ml benzamidine, and 5 mg/mL aprotinin, leupeptin, soybean trypsin

Table 1
Albumin secretion of rat hepatocytes in collagen gel sandwich and collagen gel immobilisation cultures

| Culture medium | Albumin secretion ($\mu$g/ml/24 hr) | | | | |
|---|---|---|---|---|---|
| | Collagen gel sandwich culture | | | Collagen gel immobilisation culture | |
| | +EGF | −EGF | +EGF after 2 days | +EGF | −EGF |
| Control | 15.8 ± 6.3 | 17.3 ± 5.3* | 19.9 ± 9.4 | 12.3 ± 4.8 | 12.5 ± 5.4 |
| 3.2 mM PB | 27.1 ± 3.8 | 25.4 ± 5.5 | 30.4 ± 7.0 | 19.6 ± 4.5 | 18.5 ± 6.6 |
| Control (0.05% v/v DMSO) | 14.3 ± 5.5 | 12.2 ± 4.2 | 12.7 ± 4.0 | 11.1 ± 2.8 | 11.8 ± 4.8* |
| 5 $\mu$M 3-MC | 10.9 ± 5.0 | 7.7 ± 2.6* | 8.6 ± 3.2 | 7.6 ± 4.3 | 6.7 ± 5.0 |
| 25 $\mu$M $\beta$-NF | 5.2 ± 2.0 | 5.8 ± 2.6 | 5.6 ± 1.8 | 4.2 ± 1.3 | 3.4 ± 2.1 |

Hepatocytes were cultured for 7 days either in the presence or absence of 0.02 $\mu$g/mL EGF and when 0.02 $\mu$g/mL EGF was added after 2 days for 24 hr (only in sandwich culture) and with addition of 3.2 mM PB, 5 $\mu$M 3-MC, or 25 $\mu$M $\beta$-NF when appropriate. Results are expressed (mean ± SD; N = 4), in $\mu$g albumin secreted per mL medium in 24 hr. Significant different results compared to those obtained using EGF-containing medium are indicated by *.

inhibitor. Cells were sonicated in this buffer on ice. Protein samples (150 $\mu$g) were resolved on 12.5% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Bio-Rad). Subsequently, non-specific binding sites were blocked with TBS buffer containing 4% BSA for 1 hr at room temperature. Then filters were incubated with caspase 8 antibody, 2 mg/10 mL in tris-buffered saline (TBS) containing 4% BSA. Filters were washed three times with TBS and incubated with a mouse polyclonal anti-rabbit secondary antibody conjugated to horseradish peroxidase for 1 hr at room temperature. After 4–5 washes with TBS, the proteins were visualized with Supersignal™ according the manufacturer's instructions.

### *2.13. Statistics*

Results in tables and figures are expressed as mean ± SD (N independent experiments indicated in the corresponding figures). Statistical significance between control and treated hepatocytes was tested by a paired Student's *t* test. A *P* value of $<0.05$ was considered to be statistically significant.

## 3. Results

### *3.1. Effect of EGF on albumin secretion and EROD and PROD activities*

First, we investigated whether EGF caused changes in the expression of liver specific genes in rat hepatocytes collagen I gel cultures and whether a higher response to EGF was obtained when EGF was added after 48 hr because it has previously been reported that maximal response to growth factors is obtained when stimulation is performed between 24 and 48 hr after seeding hepatocytes in conventional primary cultures [8].

Albumin secretions were significantly higher in collagen I gel sandwich culture compared to immobilisation gel culture. Addition of 20 ng/mL EGF to the different collagen gel culture media showed little effect on albumin secretion (Table 1).

In both collagen gel cultures, EROD activity values in the presence of EGF showed decreases of 35% and 27%, respectively, compared to the values measured in the absence of EGF. The PROD activity decreases were more discrete in both culture models, being 20% and 22%, respectively (Fig. 1). In EGF free medium, PB treatment of the hepatocytes caused a 7.4- and 12.8-fold increase in EROD activity, whereas only 5.1- and 8.3-fold increases were observed in the presence of EGF in collagen gel sandwich and collagen I gel immobilisation cultures, respectively (Fig. 1A). For PROD activity, a moderate effect of EGF on PB induction was observed; only in the collagen gel sandwich culture, a significant increase was measured in the absence of EGF compared to the values obtained in the EGF-stimulated cultures (Fig. 1B). In both collagen gel cultures, EGF did not have any effect on the induction of EROD activity by 3-MC and $\beta$-NF (Table 2). Addition of EGF after 2 days for 24 hr in the collagen gel sandwich cultures, induced a higher response to PB on EROD activity compared to continuous EGF supplementation, while no significant difference was observed when compared to the values obtained in PROD activity (Fig. 1). When EGF was added after 2 days for 24 hr in $\beta$-NF treated cells in the collagen gel sandwich culture, EROD activity was slightly reduced (Table 2).

### *3.2. Effect of EGF on hepatocyte cell cycle*

To determine whether in the collagen gel sandwich culture system, hepatocytes can undergo DNA replication after EGF stimulation, we measured the index of BrdU incorporation and examined expression of cell cycle genes in unstimulated and EGF-stimulated cultures. Because the collagen gel sandwich culture maintained higher levels of albumin and CYP than the collagen gel immobilisation gel culture, we further used this culture system to study the effect of EGF on hepatocyte cell cycle progression.

The BrdU labeling index was always very low in unstimulated cultures between 1 and 3% (Table 3). In EGF-stimulated cultures, this index was significantly increased reaching 12% at day 3. However, this rate of DNA replica-

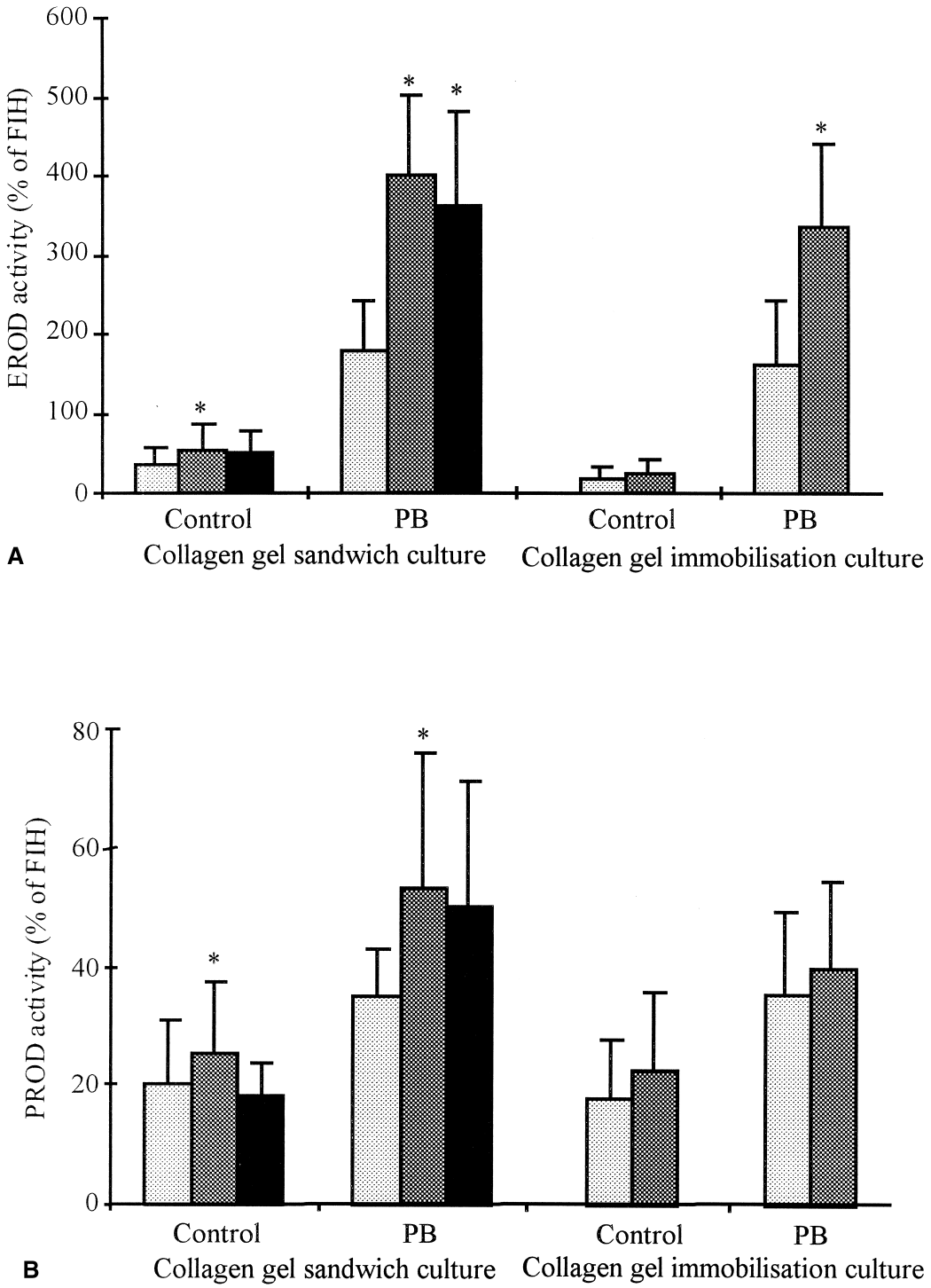


Fig. 1. CYP-dependent activity of rat hepatocytes in collagen gel sandwich and collagen gel immobilisation culture after 7 days in culture. Hepatocytes are cultured in the presence ▨ and absence ▩ of EGF (0.02 μg/mL) and when EGF was added after 2 days for 24 h ■ (only in sandwich culture) with addition of 3.2 mM PB when appropriate. A EROD activity. B PROD activity. Results are expressed as % of the value observed in freshly isolated hepatocytes (FIH), mean ± SD of 4 independent experiments. Values for FIH are 27 ± 14 pmol resorufin resorufin/mg microsomal P.min (EROD); 4.4 ± 1.4 pmol resorufin resorufin/mg microsomal P.min (PROD) *Significant different results compared to those obtained using EGF-containing medium.

tion remained much lower than those reported for conventional primary cultures (using the same techniques), which reached more than 80% [8], demonstrating that only a small fraction of hepatocytes entered S phase in collagen I gel sandwich after EGF stimulation as shown in collagen I gel monolayer cultures [9].

Table 2
Ethoxyresorufin O-deethylase activity of rat hepatocytes in collagen gel sandwich and collagen gel immobilisation culture after 7 days in culture

| Culture medium | EROD activity (% of FIH) | | | | |
|---|---|---|---|---|---|
| | Collagen gel sandwich culture | | | Collagen gel immobilisation | |
| | +EGF | −EGF | +EGF after 2 days | +EGF | −EGF |
| Control (0.05% v/v DMSO) | 61 ± 32 | 133 ± 68 | 135 ± 83 | 62 ± 51 | 81 ± 36 |
| 5 μM 3-MC | 1454 ± 1251 | 1518 ± 1288 | 1410 ± 1356 | 1232 ± 734 | 1199 ± 756 |
| 25 μM β-NF | 2394 ± 1390 | 2540 ± 1436 | 2274 ± 1422* | 1566 ± 1083 | 1677 ± 1140 |

Hepatocytes are either cultured in the presence and absence of 0.02 μg/mL EGF or when 0.02 μg/mL EGF was added after 2 days for 24 hr (only in sandwich culture) with addition of 5 μM 3-methylcholantrene or 25 μM β-naphtoflavone when appropriate. Results are expressed as % of the value observed in freshly isolated hepatocytes (mean ± SD; N = 4). Significant different results compared to those obtained in medium without EGF are indicated by *.

It has previously been reported that quiescent hepatocytes (G0 phase) spontaneously transit from G0 to G1 during isolation from liver [40] and progress in G1 without mitogenic stimulation. Then, they arrest in mid-late G1 phase and growth factors are required to induce progression in late G1 and G1/S transition [8]. Entry into and progression through G1 phase of normal hepatocytes *in vitro* and during liver regeneration, is characterized by a sequential activation of proto-oncogenes and transcription factors [41], mitogen activated protein kinases [10], cyclins and cyclin-dependent kinases (Cdks) [8,29].

To determine, in our conditions of hepatocyte isolation and culture, whether hepatocytes leave the quiescence and enter G1 phase, we analyzed the expression of immediate-early proto-oncogenes c-fos and c-jun. These two genes which have been found expressed in freshly isolated hepatocytes before to disappear in cultured hepatocytes [8,40], characterize the G0/G1 transition in hepatocytes [41].

C-*jun* and c-*fos* mRNA were also expressed in freshly isolated cells prepared with our protocol. This expression was very transient since 24 hr after plating, these two mRNAs were not detectable in hepatocytes maintained in the collagen gel sandwich system (Fig. 2A). Levels of c-myc mRNAs, a proto-oncogene expressed in early to mid-G1 phase of the hepatocyte cell cycle, were low in freshly isolated hepatocytes. This expression was strongly increased after 24 hours of culture in both unstimulated or EGF-stimulated hepatocytes (Fig. 2A).

In conventional primary cultures, cyclin D1 expression is strictly dependent on mitogenic stimulation [10] and is a limiting step for the progression in late G1 and the G1/S transition [42]. We investigated, in collagen gel sandwich cultures, whether EGF stimulation induced cyclin D1 expression which would indicate a progression in late G1 phase although only part of the hepatocytes entered S phase. Levels of cyclin D1 mRNAs were very low in both unstimulated and EGF-stimulated cultures (Fig. 2B) compared to a positive control of hepatocyte proliferation, RNAs of regenerating liver 24 hr after a two/third partial hepatectomy in which about 40% of hepatocytes are either in late G1 or S phases and express high levels of cyclin D1 [4]. We also studied the levels of Cdk1 mRNAs, which encodes the serine/threonine $p34^{cdk1}$ binding to cyclins A and B during S phase and G2/M transition of hepatocytes, respectively [29]. As previously observed with cyclin D1, levels of Cdk1 mRNAs were very low in hepatocytes maintained in collagen I sandwich compared with the level in regenerating liver (Fig. 2B).

To determine whether addition of EGF induced a slight increase of cyclin D1 expression which could correlate with the low rate of DNA replication, we exposed, for a long time, a Northern-blot analysis of cyclin D1 in unstimulated and EGF-stimulated cultures (Fig. 2C). Although the background was high, a 7- and 4.5-fold induction of cyclin D1 mRNA level was observed when EGF medium was added immediately to the culture medium or when the cells were not stimulated 48 and 72 hours of culture, respectively.

Altogether, these results demonstrate that hepatocytes isolated and maintained in our conditions of culture, left the G0 phase, entered into and progressed through early G1 phase and that only a small fraction of them entered S phase after EGF stimulation while most cells were arrested in G1.

Table 3
BrdU incorporation into DNA

| Days of culture | % of BrdU-positive cells in collagen gel sandwich cultures | |
|---|---|---|
| | −EGF | +EGF |
| 3 (80 hr) | 3 ± 1 | 10 ± 2 |
| 4 (104 hr) | <0.5 | 3 ± 1 |

Results are expressed as % BrdU positive cells, of rat hepatocytes cultured for 3 and 4 days in a collagen gel sandwich in the presence and absence of EGF (0.02 μg/mL). Each value is the mean ± SD of triplicate cultures in a typical experiment.

### *3.3. Effect of EGF on hepatocyte survival*

Conventional primary cultures of hepatocytes are characterized by a rapid decrease of liver gene expressions and cell death. Several lines of evidence indicate that hepatocyte death in conventional primary culture is mainly due to a spontaneous apoptosis involving activation of the cysteinic-aspartic proteases (caspases) 8 and 3 [12,13], two caspases

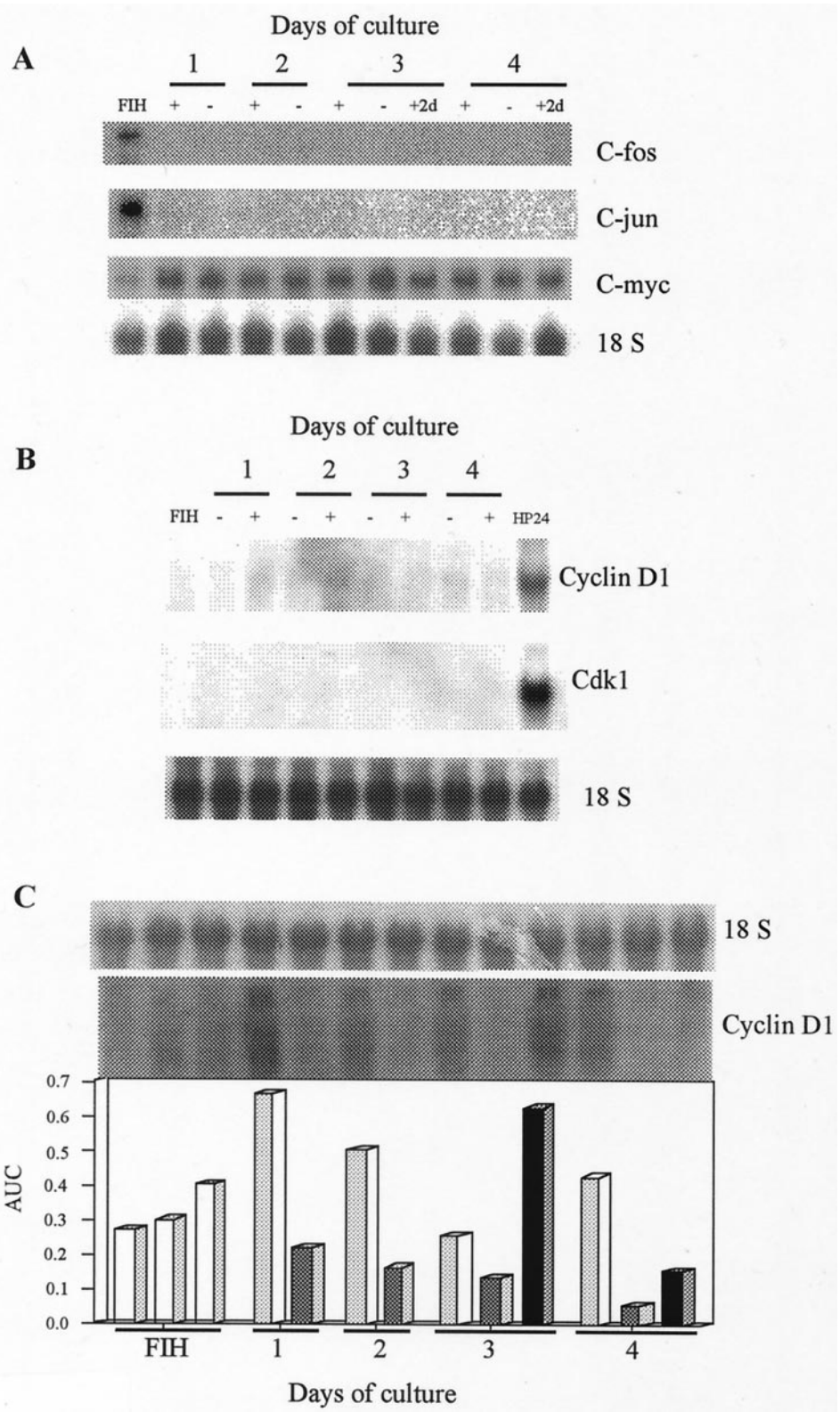


Fig. 2. (A) c-*fos*, c-*jun*, and c-*myc* mRNAs of freshly isolated rat hepatocytes and when cultured in a collagen gel sandwich culture as a function of time in the presence (+) and absence (−) of EGF (0.02 $\mu$g/mL) or when EGF was added after 2 days for 24 hr (+2d). 18S ethidium bromide staining is shown as control of RNA quality and quantity. (B) Cyclin D1 and Cdk1 mRNA in FIH of rats, regenerating liver 24 hr after a two-thirds partial hepatectomy (HP24) and rat hepatocytes cultured in a collagen gel sandwich culture as a function of time in the presence (+) and absence (−) of EGF (0.02 $\mu$g/mL). 18S ethidium bromide staining is shown as control of RNA quality and quantity. (C) Amount of cyclin D1 mRNA, expressed as area under the curve (AUC), of rat hepatocytes cultured in a collagen gel sandwich culture as a function of time in the presence ▨ and absence ▩ of EGF (0.02 $\mu$g/ml) and when EGF was added after 2 days for 24 hr ■. These data are from two distinct experiments which gave basically the same results.

involved in early and late steps of apoptosis respectively. In collagen I gel sandwich cultures, in which differentiation and survival of hepatocytes are maintained, little is known about apoptosis and activation of caspases.

To determine whether apoptosis takes place in hepatocytes maintained in collagen I gel sandwich cultures, we first analyzed activation of caspases 8 by western-blot (Fig. 3A) and then measured the DEVD-AMC caspase 3-like activity (Fig. 3B) in both conventional primary cultures and collagen I gel sandwich cultures. Procaspase 8 is present in freshly isolated cells and during all the culture time, although at higher levels in conventional culture (Fig. 3A). In collagen I gel sandwich cultures, we found the active clived form of caspase 8 expressed at very low levels at day 2. The expression of this active form of caspase 8 was strongly increased at day 3 and remained high over the 7 days of culture. Stimulation by EGF led to a slight increase of the active form of caspase 8 at day 2, then, the levels were similar in both unstimulated and EGF-stimulated cultures. In conventional primary culture, expression of active caspase 8 was very similar to that observed in collagen I gel sandwich cultures.

We also measured the DEVD-AMC caspase 3-like activity which mainly corresponds to activities of execution or caspases, involved in the late steps of apoptosis (Fig. 3B). In conventional primary culture, we have found a first and transient peak at 24 hr of culture followed by a second induction taking place at day 3 or 4 and which remained high thereafter.

In collagen I gel sandwich cultures supplemented with EGF, DEVD-AMC caspase 3-like activity was very high at day 1, significantly higher than in primary conventional cultures, strongly decreased at day 2 and remained low up to 7 days of cultures. When EGF was omitted, the DEVD-AMC caspase 3-like activity was always very low: it was significantly decreased compared to the activity in EGF-stimulated collagen I gel and conventional primary cultures at day 1 and activities in conventional primary cultures at days 5 and 7.

Altogether, these results on caspases demonstrate that: 1) a significant apoptosis occurred at day 1 in EGF-stimulated collagen I gel sandwich cultures but not in unstimulated ones, 2) neither EGF nor collagen I prevented activation of caspase 8 but collagen I induced survival signals which arrested the spontaneous apoptotic process of cultured hepatocytes downstream caspase 8 and upstream caspase 3.

## 4. Discussion

Here, we report that EGF, significantly decreases the increase of CYP1A and CYP2B1/2 activities by PB but not the 3-MC and $\beta$-NF induction of CYP1A1. However, the decrease of PB-mediated induction of CYP by EGF in the collagen I gel cultures is moderate compared to the complete inhibition of CYP1A1, 2B1/2 and 3A4 by EGF in primary conventional cultures treated with 3-MC, PB and rifampicine, respectively [6,27,28]. These results suggest that part of the inhibitory effect of EGF on CYP expressions in conventional primary cultures may be due to combined effects of EGF and phenotypic changes occurring in this model, especially, the cell cycle progression and the decrease of liver specific functions.

Regulation of cell proliferation in growth factor-stimulated hepatocytes plated on or in an extracellular matrix, is complex and related publications have reported apparent contradictory data. Rat hepatocytes cultured on Matrigel® are highly differentiated and survive for long periods of time, but they do not replicate DNA after mitogenic stimulation [43]. In contrast, Michalopoulos et al. [44] reported that in a collagen gel sandwich configuration, EGF induces DNA replication in hepatocytes, although here, the comitogen pyruvate was also added to the culture medium. However, Hansen and Albrecht [9] showed recently that hepatocytes cultured on a single collagen gel layer remain quiescent.

In our conditions of collagen I gel sandwich cultures, we showed that only a small fraction of hepatocytes replicate DNA in response to EGF stimulation. As previously observed in conventional primary cultures, we observed a rapid and transient expression of c-fos and c-jun in freshly isolated hepatocytes indicating that cells enter G1 phase during their isolation from liver. Then, we found a constant expression of c-*myc* and a weak induction of cyclin D1 in cultured hepatocytes stimulated with EGF, correlating with the low rate of replication strongly suggesting that a majority of hepatocytes were arrested in G1 phase in our culture conditions.

These results demonstrate that the decrease of PB-mediated induction of CYP by EGF is not due to a process of dedifferentiation or a consequence of the hepatocyte cell cycle progression since 3MC-mediated induction of CYP and albumin secretion are not affected and most of the hepatocytes are arrested in G1 phase. The cell cycle independent mechanism by which EGF decreases the PB-mediated induction of CYP therefore remains to be elucidated. It is a fact that specifically PB induction is difficult to obtain *in vitro* and has been shown to be inhibited by a variety of factors [11].

In this report, we also investigated the survival of hepatocytes in collagen gel cultures in presence or absence of EGF and compared the results to those obtained in primary conventional cultures maintained in the same culture medium. This study was performed by investigating the expression and activation of caspases. Recent data have demonstrated that in conventional primary cultures, hepatocyte cell death is due to a spontaneous apoptosis involving the activation of caspase 8 and 3 [12,13].

Till now, no data were available on the expression of caspases in long-term survival cultures of hepatocytes and their regulation by EGF. Unexpectedly, we demonstrate: 1) a concomitant induction of caspase 8 activation in both

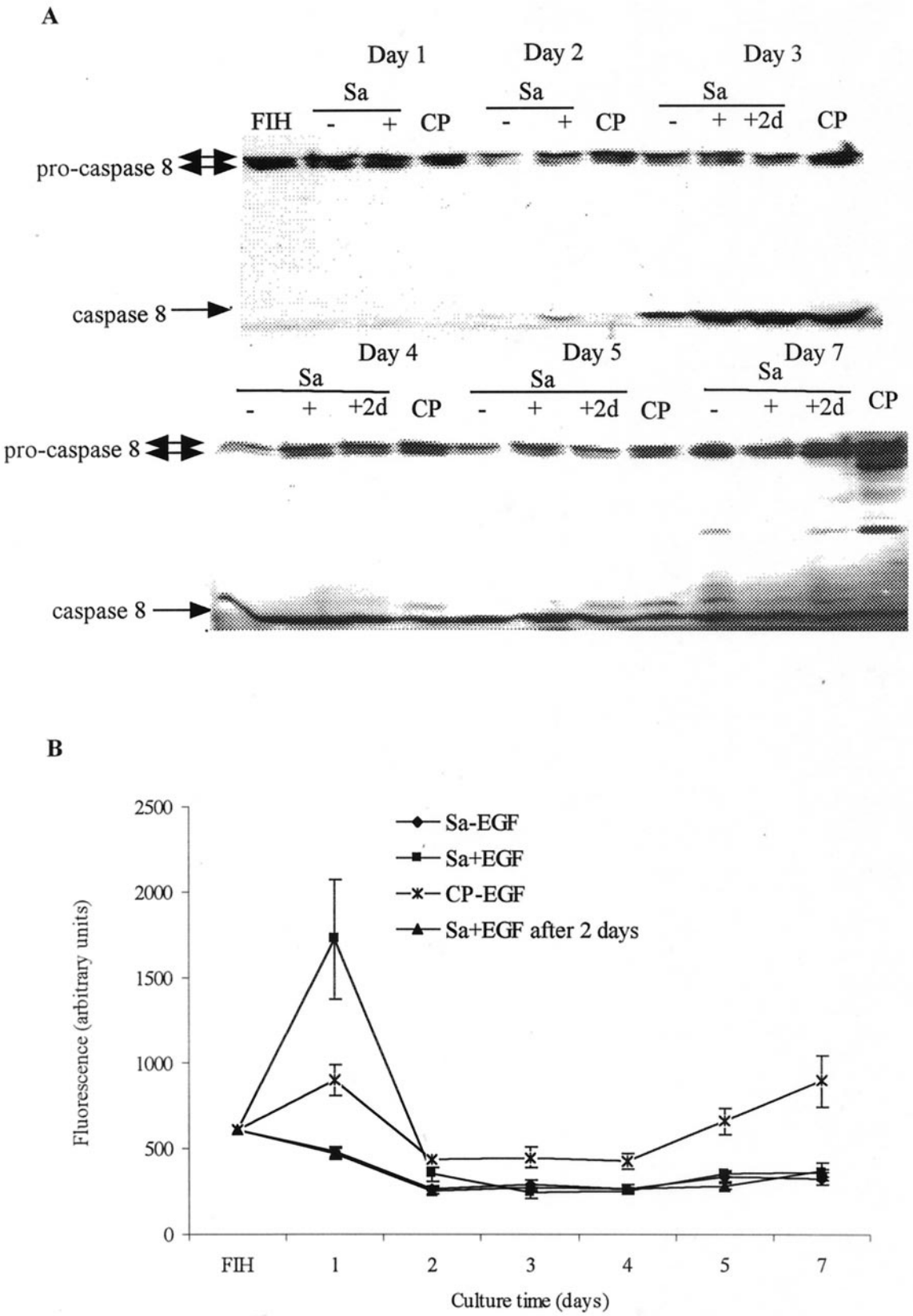


Fig. 3. (A) Caspase 8 protein of rat hepatocytes cultured in a conventional culture (CP) and a collagen gel sandwich culture (Sa) as a function of time in the presence (+) and absence (−) of EGF (0.02 μg/mL) or when EGF was added after 2 days for 24 hr (+2d). (B) DEVD-AMC caspase 3-like activity, expressed as arbitrary fluorescence units, of rat hepatocytes cultured in a conventional culture and a collagen gel sandwich culture as a function of time in the presence and absence of EGF (0.02 μg/mL) and when EGF was added after 2 days for 24 hr (2 independent experiments were performed and gave similar results).

conventional and collagen I gel hepatocyte cultures. In addition, EGF does not prevent this induction in collagen I gel cultures; 2) a transient induction of caspase 3 like activity at day 1 after plating in EGF stimulated collagen I gel and conventional primary cultures but not in collagen I gel cultures in absence of EGF; 3) the absence of late induction of the caspase 3-like activity in collagen I gel cultures 5 days after plating in contrast with its induction in conventional primary cultures.

The data presented in this report confirm previous observations that hepatocytes in conventional primary cultures commit to cell death via the activation of the caspase 8 and 3 pathway [13] and demonstrate that collagen I gel triggers two major consequences on hepatocyte phenotype: the cell cycle arrest in G1 phase of the cell cycle, as previously shown in single collagen I gel culture [9] and the inhibition of the spontaneous apoptosis downstream caspase 8 and upstream caspase 3.

These results lead us to conclude that the collagen I binding to cell surface receptors induces different intracellular signals which inhibit cyclin D1 expression [9] and caspase 3 maturation, preventing the commitment to DNA replication and apoptosis, respectively. The molecular mechanisms and signal transduction pathways may involve integrins since in various epithelial cells, binding of integrins $\alpha v\beta 3$ [44], $\alpha 5\beta 1$ [45] and $\alpha 6\beta 1$ [46] to appropriate extracellular matrix proteins can inhibit apoptosis. These survival signals activate several intracellular molecules including focal adhesion kinase [47], protein kinase B [48] and bcl-2 family members [45].

It also seems that the hepatocyte phenotype is dependent upon the physical structure and/or the abundance of the collagen I since a thin rigid film of denaturated collagen I allows hepatocyte replication after EGF stimulation [9] and does not significantly inhibit spontaneous apoptosis (Loyer, unpublished data).

Establishing optimal culture conditions is required for use of hepatocytes cultured in the collagen gel system as a model for pharmaco-toxicological purposes. The ultimate goal of optimisation of the collagen gel sandwich culture for use in pharmaco-toxicology, is to bring the hepatocytes in a "G0-like" state *in vitro* mimicking the *in vivo* situation. We confirm that hepatocytes transit from G0 to G1 phase of the cell cycle during their isolation and demonstrate that they remain arrested in G1 phase and do not return to G0 when maintained in collagen I gel sandwich cultures. However, our results indicate that an EGF-free medium is critical in achieving optimal gene responsiveness in the collagen gel sandwich culture.

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