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REGULAR ARTICLE

Co-culture of primary rat hepatocytes with rat liver epithelial cells enhances interleukin-6-induced acute-phase protein response

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Abstract Three different primary rat hepatocyte culture methods were compared for their ability to allow the secretion of fibrinogen and albumin under basal and IL-6-stimulated conditions. These culture methods comprised the co-culture of hepatocytes with rat liver epithelial cells (CC-RLEC), a collagen type I sandwich culture (SW) and a conventional primary hepatocyte monolayer culture (ML). Basal albumin secretion was most stable over time in SW. Fibrinogen secretion was induced by IL-6 in all cell culture models. Compared with ML, CC-RLEC showed an almost three-fold higher fibrinogen secretion under both control and IL-6-stimulated conditions. Induction of fibrinogen release by IL-6 was lowest in SW. Albumin secretion was decreased after IL-6 stimulation in both ML and CC-RLEC. Thus, cells growing under the various primary hepatocyte cell culture techniques react differently to IL-6 stimulation with regard to acute-phase protein secretion. CC-RLEC is the preferred method for studying cytokine-mediated induction of acute-phase proteins, because of the pronounced stimulation of fibrinogen secretion upon IL-6 exposure under these conditions.

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Abbreviations

CC-RLEC	Co-culture of primary hepatocytes with rat liver epithelial cells
DMEM	Dulbecco's Modified Eagle's Medium
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
IL-6	Interleukin-6
ML	Monolayer hepatocyte cell culture
PBS	Phosphate-buffered saline
RLEC	Rat liver epithelial cells
SW	Sandwich culture of primary hepatocytes with collagen

Introduction

The acute hepatic response to systemic injury has been described as a re-prioritisation of liver protein synthesis: the available synthetic capacity and amino acid resources are shifted from constitutive protein production, e.g. albumin and transferrin, to increased non-constitutive acute-phase protein production, e.g. fibrinogen and haptoglobin. The last two mentioned are designated as positive acute-phase proteins and play important roles in the restoration of homeostasis after injury and metabolic stress. Acute-phase protein synthesis is induced by a multitude of stress factors,

such as cytokines and stress hormones (Baumann and Gauldie 1990, 1994; Moshage 1997), with the main inducer being interleukin-6 (IL-6; Castell et al. 1989; Gauldie et al. 1992; Heinrich et al. 1990).

Most in vitro studies on the acute-phase protein response have been carried out on a human hepatocellular cell-line, viz. HepG2 cells (ATCC number HB-8065; Derfalvi et al. 2000; Giri et al. 1992; Shen et al. 2000; Ulrich et al. 1999; Verselis et al. 1999). However, the HepG2 cell line is a hepatocellular carcinoma cell line and might exhibit different metabolic properties from those observed in primary hepatocytes. Therefore, an investigation of the effects of IL-6 on acute-phase protein synthesis in primary hepatocytes is of interest.

Primary hepatocyte cultures have been used successfully to study detoxification processes by cytochrome P450 activity (Donato et al. 1993; Rogiers and Vercruysse 1998) and to a minor extent to study acute-phase protein expression (Ikawa and Shozen 1990; Talamini et al. 1998). Monolayer hepatocyte cultures have also been used to study cytokine-induced acute-phase protein expression in rat (Sosef et al. 2005) and human (Castell et al. 1990; Clement et al. 1984) hepatocytes.

In addition to cytokines, the extrahepatocellular environment might play a role in the establishment of the acute-phase response in primary cell cultures. Whether the differentiated state of cultured hepatocytes is maintained over time depends on a complex environment in which exogenous factors and cell-matrix and cell-cell interactions play key roles (Guillouzo et al. 1990; Rogiers and Vercruysse 1993). These conditions can be mimicked by co-culturing hepatocytes with non-parenchymal rat liver epithelial cells of primitive biliary origin in order to mimic the in vivo situation in which hepatocytes also make contact with liver epithelial cells via the perisinusoidal space of Disse (Rogiers and Vercruysse 1998; Vanhaecke et al. 1998). Results from Guillouzo et al. (1984) suggest that this method might improve acute-phase protein synthesis. A second approach to providing an extracellular matrix is the addition of a collagen bilayer. Type I rat tail collagen is generally used as the coating material for hepatocyte cultures, as it is an important component of the hepatocytic basal membrane (Beken et al. 1998; Muller et al. 2004; Wang et al. 2004).

We have used three different cell culture techniques in order to investigate the effects of the extracellular environment on acute-phase protein synthesis in vitro. In particular, we have studied the effect of IL-6 on the expression of fibrinogen (a positive acute-phase protein) and albumin (a constitutively expressed protein and/or negative acute-phase protein) by a conventional primary monolayer culture of hepatocytes (ML), a co-culture of hepatocytes with rat liver epithelial cells (CC-RLEC) and a collagen type I sandwich culture (SW). This study is the first approach to compare the effects of IL-6 stimulation on fibrinogen and albumin secretion in three different cell culture models of hepatocytes from the same batch.

Materials and methods

Chemicals

Crude collagenase type I, bovine serum albumin fraction V, insulin and L-glutamine were purchased from Sigma (St. Louis, Mo., USA). William's medium E (without L-glutamine), fetal bovine serum (FBS) and trypsin-EDTA solution were from Invitrogen (Brussels, Belgium). Hydrocortisone was from Upjohn (Puurs, Belgium).

Animals

Adult male outbred Sprague-Dawley rats (±200 g; food and water *ad libitum*; Iffa Credo, Brussels, Belgium) were anaesthetised by intraperitoneal injection of sodium pentobarbital solution (0.1 ml/100 g body weight).

Isolation and culture of hepatocytes

Intact rat hepatocytes were isolated by collagenase perfusion according to De Smet et al. (1998). After being tested for cell integrity by trypan blue exclusion, 1.6×10^6 hepatocytes were cultured in 4 ml DMEM containing 10% FBS (v/v), 2.5 µg/ml bovine insulin, 0.005% kanamycin monosulphate, 0.005% streptomycine sulphate and 0.0045% penicillin on 6-cm diameter culture dishes as ML (Vanhaecke et al. 1998), CC-RLEC (Vanhaecke et al. 1998) or SW (Beken et al. 1998). Co-cultures were set up, after 4 h, by adding 25% of a confluent flask (75 cm^2) containing epithelial cells. These were isolated from livers of 8-day-old Sprague-Dawley rats and cultured as described by Henkens et al. (2006). Media were supplemented with 7×10^{-5}M or 7×10^{-6}M hydrocortisone hemicuccinate for replenishment of ML and CC-RLEC, respectively. After 24 h, the cells were incubated with 10 ng/ml recombinant rat IL-6 (Peprotech, Heerhugowaard, The Netherlands). Both IL-6 and the medium were refreshed every 24 h. After 24 and 48 h, media samples were taken and analysed for the amount of albumin and fibrinogen secreted over 24 h. All experiments were carried out three times (n=6).

Enzyme-linked immunosorbent assay for fibrinogen and albumin

For enzyme-linked immunosorbent assay (ELISA), sheep anti-rat fibrinogen purified IgG and sheep anti-rat fibrino-

gen conjugated to horseradish peroxidase (HRP) were purchased from Affinity Biologicals (Ancaster, Ontario, Canada), fibrinogen from rat plasma was from Sigma and sheep anti-rat albumin, sheep anti-rat albumin HRP-labelled and rat albumin antigens were from Biogenesis (Pool, England). A 96-well flat bottom EIA/RIA plate (Corning Life Sciences, Schiphol-Rijk, The Netherlands) was coated overnight with primary antibody diluted in phosphate-buffered saline (PBS). Plates were washed after every step with 0.1% (v/v) Tween-20 (Merck Eurolab, Roden, The Netherlands) in 0.1 M PBS pH 7.4. Subsequently, the plates were blocked with 5 % (w/v) Protifar (Nutricia, Zoetermeer, The Netherlands) in 0.1 M PBS pH 7.4 for 90 min. Samples and antigen were then incubated in 0.1% (v/v) Tween-20 in 0.1 M PBS pH 7.4 for 90 min. Subsequently, the plates were incubated with HRP-conjugated antibodies in 0.1% (v/v) Tween-20 in 0.1 M PBS pH 7.4 for 90 min. Finally, a colorimetric reaction was carried out with 100 µl undiluted 1-Step Ultra TMB-ELISA (Pierce, Rockland, Ill., USA). The reaction was stopped with 50 µl 2 M sulphuric acid and absorbance was measured at λ=450 nm.

Protein assay

Protein levels of the cell lysates were measured by using a Bio-Rad Protein Assay Kit (Bio-Rad, Brussels, Belgium) with bovine serum albumin as a standard. To be able to correct for protein content in RLEC, the percentage of RLEC was determined as follows. After the hepatocytes had been detached from the epithelial cells with 4 ml collagenase in PBS buffer (1 mg/ml) at 37°C, the remaining RLEC were scraped off and subjected to the protein assay. The percentage of RLEC in co-culture was estimated by calculating the ratio of the protein content of the RLEC in co-cultures versus the protein content of the hepatocytes plus the RLEC.

Statistics

To determine possible significant differences between values, multiple pairwise comparisons were conducted with Student's *t*-test. *P*-values below 0.05 were interpreted as statistically significant.

Results

Hepatocyte morphology in ML, CC-RLEC and SW

Hepatocyte morphology in ML, CC-RLEC and SW after 24-h incubation are shown in Fig. 1a–c. In ML, hepatocytes cluster on the culture dish (Fig. 1a). In the CC-RLEC, the cells also are clustered but the RLEC fill in the empty spaces on the culture dishes (Fig. 1b), whereas in the SW, the cells are present between a collagen bilayer, with the cells on all sides lying in contact with collagen as the extracellular matrix (Fig 1c).

Effects of IL-6 on fibrinogen secretion

Fibrinogen secretion in the media of the different cell cultures after a 24-h incubation with IL-6 is shown in Fig. 2. The basal fibrinogen secretion is significantly higher in CC-RLEC than in the other cell culture models ($P<0.0001$). The basal secretion of fibrinogen in SW and ML were comparable. When incubated with IL-6, both ML ($P<0.001$) and CC-RLEC ($P<0.001$) showed an approximately three-fold increase in fibrinogen secretion compared with basal. In contrast, the effect of IL-6 on the fibrinogen secretion in SW was minor but nevertheless significant ($P<0.01$). The IL-6-induced fibrinogen secretion of CC-RLEC was significantly higher ($P<0.00001$) versus ML, whereas the IL-6 response in SW was significantly lower ($P<0.0001$) than that in ML.

Effects of IL-6 on albumin secretion

In Fig. 3, the basal albumin secretion rates of the hepatocyte cultures are shown to be similar in all cell cultures. When incubated with IL-6, albumin secretion in both ML and CC-RLEC decreased significantly ($P<0.01$) and to the same extent. The response to IL-6 was negligible in SW. As a control, albumin secretion was also determined in the RLEC in a separate study (without hepatocytes). No albumin was present in the media of the RLEC. In addition, in earlier experiments, we did not find albumin expression in the RLEC as determined by immunostaining (Snykers et al. 2007).

Stability and reproducibility of the cell cultures

In order to determine the stability and reproducibility of measurements in the cell cultures, all incubations were extended for an additional 24-h period. The albumin secretion after two consecutive days in the different cell cultures is shown in Table 1. Both ML and CC-RLEC showed a significant decrease of albumin secretion in both basal and IL-6-stimulated conditions, whereas in SW, secretion levels of albumin did not change. Similar results were observed for fibrinogen secretion. ML and CC-RLEC showed a decrease in basal fibrinogen secretion rates, whereas basal fibrinogen secretion remained constant in SW (Table 2). The capacity of IL-6 to induce fibrinogen secretion or to reduce albumin secretion was also maintained in ML and CC-RLEC after 48-h incubation.

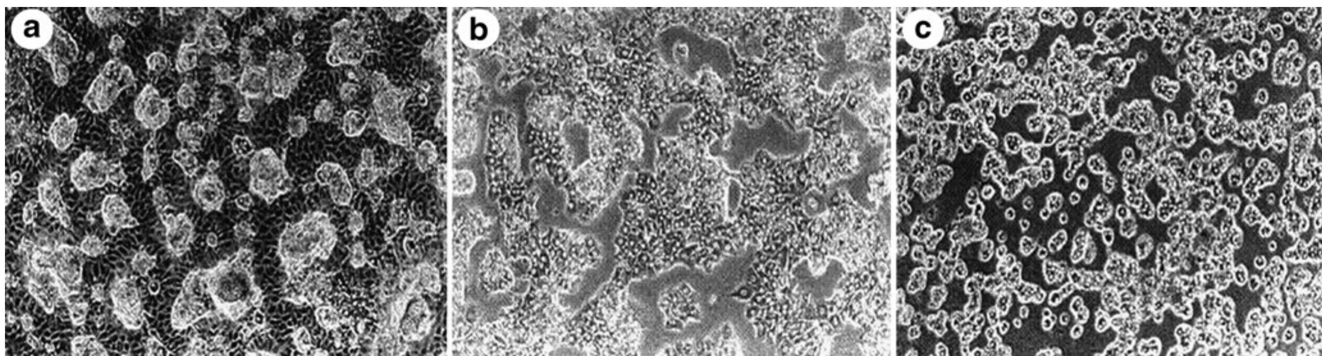

Fig. 1 **a** Hepatocytes in monolayer cell culture (ML). **b** Co-culture of hepatocytes and rat liver epithelial cells (CC-RLEC). **c** Collagen sandwich culture (SW) of hepatocytes. ×100

Discussion

In the present study, the effects of various culture conditions on the secretion of albumin and fibrinogen by primary hepatocytes of the same batch have been studied under basal and IL-6-induced conditions. IL-6 is the most important inducer of the acute-phase protein response (Baumann and Gauldie 1994; Heinrich et al. 1990). Fibrinogen is an inducible acute-phase protein and, quantitatively speaking, one of the most abundant acute-phase proteins (Fleck 1989). Fibrinogen secretion is induced by IL-6 (Castell et al. 1989; Derfalvi et al. 2000; Gauldie et al. 1990; Marinkovic et al. 1989).

Basal and IL-6-stimulated fibrinogen secretion is higher in CC-RLEC than in ML. This might be attributable to increased secretion by the hepatocytes or to additional secretion by the epithelial cells. Lawrence and Simpson-Haidaris (2004) have shown that extrahepatic epithelial cells are also able to secrete fibrinogen under inflammatory conditions, but not under basal conditions. Our results demonstrate that fibrinogen secretion under both control and inflammatory conditions is increased. α_2-Macroglobulin is another positive acute-phase protein. Talamini et al. (1998) have found that the interaction between hepatocytes and endothelial cells in co-culture induces the expression of α_2-macroglobulin; they conclude that endothelial cells can modulate hepatocyte acute-phase gene expression by cell-cell contact. Moreover, in co-culture with epithelial cells, the acute-phase protein responses of hepatocytes might be more pronounced (Guillouzo et al. 1984). Our results confirm these observations, in that epithelial cells can also modulate both the basal and the acute-phase protein response of fibrinogen, since both basal and IL-6-induced fibrinogen excretion in CC-RLEC is significantly induced when compared with that in ML. Whether the increased fibrinogen synthesis is caused by cell-cell interactions of hepatocytes and epithelial cells, by fibrinogen secretion by the epithelial cells or by mutual stimulation via soluble factors remains to be investigated.

The IL-6 induced secretion of fibrinogen in SW is marginal but significant. The induction of fibrinogen secretion by IL-6, however, is significantly less than that

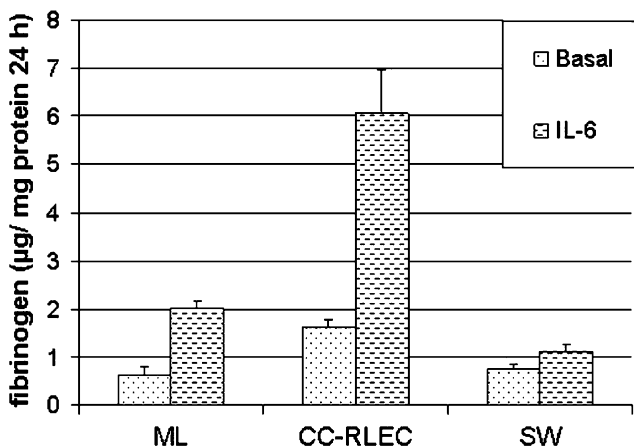

Fig. 2 Fibrinogen secretion of primary rat hepatocyte cultures (*n*=6). Control versus IL-6 (10 ng/ml)-stimulated hepatocytes (*ML* monolayer, *CC-RLEC* hepatocytes co-cultured with rat liver epithelial cells, *SW* collagen type I hepatocyte sandwich culture)

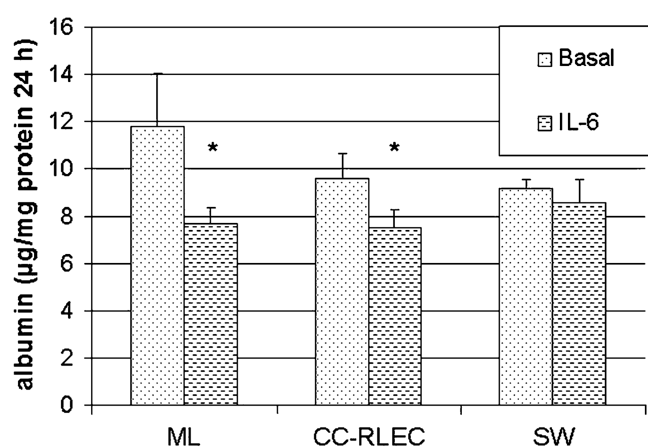

Fig. 3 Albumin secretion by primary rat hepatocyte cultures (*n*=6). Control versus IL-6 (10 ng/ml)-stimulated hepatocytes (*ML* monolayer, *CC-RLEC* hepatocytes co-cultured with rat liver epithelial cells, *SW* collagen type I hepatocyte sandwich culture). *P<0.01 versus basal

Table 1 Stability of albumin secretion in three types of cell culture after 24-h and 48-h incubations (*n*=6)

Culture type	Albumin secretion (µg/mg protein)			
	24 h		48 h	
	Basal	With interleukin-6 (10ng/ml)	Basal	With interleukin-6 (10ng/ml)
Monolayer	11.8±2.2	7.7±0.7	7.4±1.9*	2.5±1.4**
Hepatocytes co-cultured with rat liver epithelial cells	9.6±1.1	7.5±0.8	6.2±1.2*	3.6±0.6**
Collagen type I hepatocyte sandwich culture	8.2±0.4	8.6±1.2	8.2±1.3	8.1±1.0

**P*<0.01 versus 24 h Basal

***P*<0.01 versus 24 h with interleukin-6

in ML. Earlier experiments have shown that cell-cell interactions between hepatocytes in collagen bilayer cultures are critical with regard to albumin synthesis (Moghe et al. 1997). Basal albumin and fibrinogen secretions are not affected by the collagen bilayer *per se* in the SW when compared with ML. Hence, cell conditions seem to be the same in ML and SW cultures. Another possible explanation for the minimal effects of IL-6 in SW might be the physical barrier that is created by the collagen bilayer of the culture, which may cause a decreased interaction of IL-6 with the cells or a decreased secretion of albumin or fibrinogen. However, the latter does not seem to be the case, since albumin and fibrinogen secretions are not inhibited by the collagen bilayer, when compared with ML, as mentioned above.

In both ML and CC-RLEC, albumin has turned out to be a negative acute-phase protein, i.e. its synthesis decreases after IL-6 exposure. These findings are in accordance with the classic literature, which refers to albumin as being a negative acute-phase protein (Ritchie et al. 1999a, 1999b). Separate studies have indicated that RLEC in monoculture (without hepatocytes) are unable to secrete albumin (our results; Snykers et al. 2007). A decrease rather than an increase in albumin secretion has been observed in CC-RLEC. Taken together, the decreased albumin secretion in combination with the increased fibrinogen secretion suggests that the hepatocytes are the main producers of both acute-phase proteins.

In cancer cachexia, however, the observed hypoalbuminemia has been reported not to be linked to a decrease in the hepatic albumin synthetic rate (Fearon et al. 1998; Mansoor et al. 1997). This finding casts doubt on the idea of albumin being a negative acute-phase protein. Nevertheless, the in vitro experiments of Mackiewicz et al. (1991) on a human hepatocellular cell line (HepG2 cells) have demonstrated reduced albumin secretion. Moreover, Kasza et al. (1994) have reported a negative effect of IL-6 on albumin synthesis in long-term experiments on HepG2 cells. Our findings also confirm the hypothesis of albumin being a negative acute-phase protein. These contradictory observations under in vivo and in vitro conditions might be explained on the basis that, in the in vivo situation, a range of factors affect and mediate the hepatic response to stress, such as the many types of cytokines and hormones that are present during the stress response.

The co-culture of hepatocytes with Kupffer cells (West et al. 1985, 1986), Ito cells (Lonberg-Holm et al. 1987; Rojkind et al. 1995) or RLEC (Goulet et al. 1988; Guillouzo et al. 1984) has demonstrated the prolonged maintenance of specific hepatic functions by hepatocytes.

Table 2 Stability of fibrinogen secretion in three cell cultures after 24 and 48-h incubations (*n*=6)

Culture type	Fibrinogen secretion (µg/mg protein)			
	24 h		48 h	
	Basal	With interleukin-6 (10ng/ml)	Basal	With interleukin-6 (10ng/ml)
Monolayer	0.62±0.16	2.0±0.16	0.26±0.04*	1.29±0.13**
Hepatocytes co-cultured with rat liver epithelial cells	1.63±0.18	6.1±0.94	0.85±0.08*	2.63±0.30**
Collagen type I hepatocyte sandwich culture	0.74±0.11	1.1±0.16	0.98±0.07***	1.26±0.16

*Decreased when compared with 24 h Basal (*P*<0.01)

**Decreased when compared with 24 h interleukin-6 (*P*<0.01)

***Increased when compared with 24 h Basal (*P*<0.01)

Two potential mechanisms have been proposed to explain these findings: (1) intercellular communication mediated by gap junctions (Goulet et al. 1988; Rojkind et al. 1995) and (2) synthesis of extracellular matrices (Guillouzo et al. 1984; Loreal et al. 1993). Moreover, rat hepatocyte proliferation is inhibited when co-cultured with epithelial cells (Wegner et al. 1992). This might be attributable to secreted growth inhibitors by the epithelial cells. This inhibition of proliferation might be responsible for the maintenance of cell differentiation and, hence, the attenuated ability of hepatocytes to secrete fibrinogen and albumin under CC-RLEC or SW conditions.

In conclusion, both ML and CC-RLEC are suitable conditions for studying acute effects of cytokines on acute-phase protein secretion. In contrast, SW is less preferable, because the interaction of IL-6 with the hepatocytes may be impaired by the collagen barrier. When extracellular rat liver epithelial cells are added to hepatocytes, as in CC-RLEC, basal fibrinogen secretion is significantly higher than under ML conditions. These differences can partly be explained by the properties of the epithelial cells, which might include the ability to synthesise fibrinogen or cytokines. An extracellular matrix *per se* does not seem to be the explanation for the increased basal fibrinogen expression, since an increase of fibrinogen synthesis is not observed in SW in which an extracellular matrix is provided by a collagen type I bilayer. In addition, although SW is less sensitive to IL-6 stimulation, the production of albumin over time is more stable. We have compared three rat hepatocyte cell culture techniques on one batch of primary hepatocytes with regard to IL-6 induced acute-phase protein synthesis. In future studies, the effects of the RLEC on acute-phase protein synthesis need to be investigated to determine the role of RLEC in increased basal fibrinogen secretion. Nevertheless, since fibrinogen induction is most pronounced in the CC-RLEC, we consider CC-RLEC to be the preferred method for studying the effects of IL-6 on acute-phase protein synthesis in primary hepatocytes, rather than ML and SW.

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