Histol Histopathol (2007) 22: 399-407

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Short term regulation of hepatocyte glutathione content by hepatic sinusoidal cells in co-culture

M. Catalá[1], R. Pagani[2] and M.T. Portolés[2]

[1]Department of Natural Sciences and Applied Mathematics, Higher School of Experimental Science and Technology, Universidad Rey Juan Carlos, Spain and [2]Department of Biochemistry and Molecular Biology I, Faculty of Chemistry, Universidad Complutense de Madrid, Spain

Summary. An inadequate balance between oxidant species and antioxidant mechanisms may constitute the primary mechanisms of a number of pathologies. The liver plays a central role in this balance: parenchymal hepatic cells contain and export especially high levels of the antioxidant glutathione and activated Kupffer cells release inflammation mediators and reactive oxygen species. There is growing evidence of a paracrine regulation of hepatic function by means of a fluent intercellular communication which must still be fully elucidated, especially in basal conditions. *In vivo* models provide often too complex results but, *in vitro*, tissue interactions are left aside; therefore it is important to find new experimental models to address cell communication studies. Here we propose the complementary use of three models to study liver glutathione system regulation in basal conditions: pure parenchymal cells primary cultures, addition of sinusoidal cell conditioned media to parenchymal cells and co-culture of sinusoidal cells using porous membranes. We have also developed a high specifity immunofluorescent method for the complete characterization of sinusoidal cell populations by flow cytometry and confocal microscopy. Our results show that Kupffer cells possess higher levels of reactive oxygen species than sinusoidal endothelial cells even in basal conditions. We also report that the glutathione content of hepatic parenchymal cells in basal conditions is regulated by a sinusoidal-parenchymal cells cross-talk and suggest the existence of a paracrine circuit in the management of liver oxidative stress.

Key words: Glutathione, Co-culture, Hepatocyte, Kupffer cell, Sinusoidal endothelial cell

Offprint requests to: Myriam Catalá Rodríguez, ESCET. Dptal I, 241, Universidad Rey Juan Carlos, c/ Tulipán s/n, E-28933 Móstoles, Spain. e-mail: myriam.catala@urjc.es

Introduction

A great number of pathophysiological processes are accompanied by the release of free radicals that initiate lipid peroxidation events usually inducing irreversible alterations on cell membranes and organ disfunction (Halliwell and Gutteridge, 1984; Portoles et al., 1993; Halliwell, 1994). The survival of cells in this environment would not be possible without powerful defence mechanisms against oxidant species. Among these mechanisms the tripeptide glutathione stands out, being the most important cellular low molecular weight thiolic compound and the main reducing agent in cells due to the antioxidant power of the thiolic group in its cysteine (Forman and Fisher, 1981; Meister and Anderson, 1983). Parenchymal hepatic cells contain and export especially high levels of glutathione, which makes them a key point in response to free radicals. An inadequate balance between oxidant species and antioxidant mechanisms may constitute the primary mechanisms of a number of pathologies (Wohaieb and Godin, 1987; Halliwell, 1994; Taylor and Piantadosi, 1995; Cesaratto et al., 2004).

Sinusoidal cells deeply regulate the hepatic function, turning the liver into the major organ of the reticulo-endothelial system (RES) responsible for the unspecific inflammatory response. RES has an important paracrine action on hepatic function. There is growing evidence of the paracrine regulation of protein or glucose liver metabolism by means of a fluent intercellular communication, especially evident in pathological states. Activated Kupffer cells release inflammation mediators such as TNF-α, IL-1, IL-6, IL-10, IFN-γ, PGs, NO and reactive oxygen species (reviewed in Kmiec, 2001) that can cause damage to other tissues (Cogger et al., 2004; Cesaratto et al., 2004). The parenchymal cells activated by these cytokines produce an acute phase response with the release of NO, among other metabolites, which is involved in Kupffer cells' inflammatory response (Harbrecht and Billiar, 1995).

In vivo experimental models provide valuable but complex results. The use of *in vitro* approaches, such as

primary cell cultures, gives more easily interpretable results, but conclusions are difficult to extrapolate since tissue interactions are left aside. These facts suggest the importance of finding new experimental models complementing pure primary culture studies. Tafazoli et al. (2005) remarks the need for new models representing an intermediate step bridging the gap between *in vitro* and *in vivo* models in toxicological studies. One of these intermediate models is the addition of sinusoidal cell conditioned media to cultured parenchymal cells. One step further is the co-culture of both cell types using porous membranes, since it allows cellular communication through the diffusion of mediators in the culture medium (McMillan, 1995; Yerkovich, 2004; Balafa, 2005; Matsuo, 2005). This model allows, for instance, studying the effects of intercellular communication between activated sinusoidal cells and non activated parenchymal cells. Using porous membrane inserts both cell populations can be manipulated independently before and after the experiment.

In the present work three models have been used to study liver glutathione system regulation in basal conditions: 1) Pure parenchymal cells primary cultures. 2) Addition of sinusoidal cells conditioned media to pure parenchymal cell cultures. 3) Co-culture of sinusoidal and parenchymal cells using porous membranes.

Hepatic sinusoidal cells have been widely used in liver cell communication studies (Kuiper et al., 1988; Curran et al., 1989; Billiar et al., 1990b; Monden et al., 1991; DeLeve et al., 1997; Cutrin et al., 1998; Hamada et al., 1999; Fukuda et al., 2004). The sinusoidal cells population is composed of 2 cells types of very different origin and function: sinusoidal endothelial cells and Kupffer cells, the liver resident macophages. Purification and characterization of these populations are not simple since both cell types present similar size and density in suspension. Therefore we have also developed an immunostaining method with a fluorescent probe for the complete characterization of sinusoidal cell populations by flow cytometry and confocal microscopy.

Material and Methods

Materials

Collagenase and pronase were obtained from Boehringer-Mannheim Diagnostica (Mannheim, Germany). Nycodenz was from Nycomed AS (Oslo, Norway). Monoclonal antibody ED1 conjugated to PE (MCA34LP) was supplied by Serotec (Bicester, UK). Ham's F-12, L-glutamine and antibiotics were from Seromed, Biochrom (Berlin, Germany). Foetal calf serum was from Biowhitaker (Francia). Dulbecco's Minimal Essential Medium (DMEM) with 25mM HEPES, propidium iodide (PI), William's E medium, 5,5'-Dithio-bis(2-nitrobenzoic acid) (DTNB), glutathione reductase (GR), glutathione reduced form (GSH), vinylpiridine and azide were obtained from Sigma Chemicals (St.Louis, MI, USA). Nicotinamide adenine dinucleotide phosphate, reduced form (NADPH) was from Boehringer Manheim (Germany). 2',7'-dichlorofluorescein diacetate (DCFH/DA) was purchased from Serva (Heidelberg, Germany). Other chemicals were purchased from Merck (Darmstad, Germany). Costar Transwell, permeable polycarbonate membrane (pore diameter 0.4 μm) wells were used for the co-cultures. Pentothal-Na was purchased to Abbot International Ltd (USA).

Animals

Hepatic cells were obtained from male Wistar rats (150-200 g body weight, fasted overnight with water *ad libitum*). The cell isolation procedures were performed in adherence to the EU (86/609) and Ministerio de Agricultura (Spain, BOE 223/1988) guidelines for care and use of laboratory animals. Every animal was initially anesthetised with ether and then injected intra-peritoneally with 100 mg Pentothal-Na / 100 g weight.

Isolation and culture of hepatocytes

Liver parecchymal cells (HCS) were isolated by the perfusion technique, using collagenase in Krebs-Ringer-bicarbonate solution (KRB medium) according to the general method of Berry and Friend (Berry and Friend, 1969) as described previously (Portoles et al., 1987; Catala and Portoles, 2002a). Cell viability was routinely monitored by the Trypan blue exclusion test obtaining 85-90% viability. Yield of viable HCS was (235±22) x 10^6 per rat liver with a purity above 99%. The isolated HCS were cultured in William's E medium in the presence of 10% foetal calf serum, with 500 U/ml penicillin, 100μg/ml streptomycin, at 37ºC under a CO_2 atmosphere for 24 h, up to the formation of a continuous monolayer, and were then used. No collagen or any other coating was used. The cells were cultured on either 25 cm^2 flasks or on 4.5 cm^2 Costar Transwell®, membranes ($2.8x10^5$ cells/cm^2). Cell culture was performed under sterile conditions in a Telstar CAM 7001 laminar flow chamber.

Isolation and culture of sinusoidal cells

Liver sinusoidal cells (Sc) were isolated by perfusion with collagenase and pronase in Gey's balanced salt solution according to the method of Wisse and Knook (Wisse and Knook, 1977) with minor modifications (Catala et al., 1999). After preparation of the sinusoidal cell suspension with a single-step Nycodenz gradient, Kupffer cells were further purified by centrifugal elutriation at 4ºC in a JE-6B rotor equipped with a standard separation chamber, using a Beckman model J-6B centrifuge (Beckman Instruments, Fullerton, CA), as previously described (Gressner and Zerbe, 1987; Catala et al., 1999). Cell viability was routinely monitored by the Trypan blue exclusion test or flow cytometry after the addition of PI (0.005% in phosphate buffer saline), obtaining 85-90 % viability.

Yield of viable sinusoidal cells was (67±20) x 10^6 per rat liver with a purity above 99%. Sinusoidal cell suspensions were constituted by 30% Kupffer cells and 70% sinusoidal endothelial cells as evaluated by flow cytometry (Catala et al., 1999). The isolated Sc were cultured in Ham's F-10 medium in the presence of 10% foetal calf serum, at 37ºC in either 25 cm^2 flasks or 9.5 cm^2 Costar multiwell (10^5 cells/cm^2) under a CO_2 atmosphere for two weeks. No collagen or any other coating was used. Cell culture was performed under sterile conditions in a Telstar CAM 7001 laminar flow chamber.

Immunolabelling and characterization of Kupffer cells by flow cytometry and confocal microscopy

Kupffer cells (Kc) in suspension were labelled with the monoclonal antibody ED1, which specifically recognizes rat macrophages, as previously described in detail (Catala et al., 1999). Briefly, Kupffer cells were sedimented at 208xg for 10 min, suspended in PBS 1% foetal calf serum and incubated for 30 min with ED1 conjugated to PE (10 μl ED1-PE/10^5 cells) and protected from light onwards. Cells were then diluted 100-fold with buffer, sedimented and resuspended in DMEM with 25 mM HEPES (10^6 cells/ml). Alter labelling of Kc population with ED1-PE, cell suspension was studied exciting the fluorescence of PE linked to ED1 at 488nm and measuring the emitted fluorescence at 575nm in a FACStar Plus Cytometer. The same immunolabelling procedure was adapted to characterize Kc in monolayers of cultured liver sinusoidal cells and the fluorescence of PE linked to ED1 was observed with a Bio-Rad 1024 confocal microscope (Centro de Citometría de Flujo, Universidad Complutense de Madrid).

Intracellular reactive oxygen metabolite study by confocal microscopy

Sinusoidal cells cultures were incubated at 37º C for 30 min with 10 μM 2',7'-dichlorofluorescein diacetate (DCFH/DA) in a modified Krebs-Ringer buffer (Portoles et al., 1994). DCFH/DA enters the cell where it is transformed by intracellular esterases to DCFH and this product yields a fluorescent DFC if oxidized by free radicals. This method is not quantitative but gives a rough estimate of ROS produced by the cells. The fluorescence of intracellular DCF was observed with a Bio-Rad 1024 confocal microscope (Centro de Citometría de Flujo, Universidad Complutense de Madrid).

Treatment of cultured hepatocytes with sinusoidal cells´conditioned medium

Cultured sinusoidal cells were incubated for 0, 15 or 120 min with fresh medium. Supernatants were then gathered and immediately transferred onto HCS previously washed twice with PBS to remove cellular debris. Cultured HCS remained in Sc conditioned media for various times, and then incubation medium was aspirated and HCS monolayers were washed twice with PBS, and frozen at -80ºC. The integrity of the monolayer was monitored by contrast-phase microscopy before and after each experiment. For each experiment one rat was used for the isolation and growth of Sc and another one for the isolation of HCS.

Co-culture of parenchymal and sinusoidal cells

Sc cultured on 9.5 cm^2 Costar multiwells were washed twice with PBS and 4.5 ml medium was added. HCS cultured on Costar Transwell, membranes were also washed twice with PBS and placed into the wells containing Sc (Fig. 1). Then 1.5 ml medium was added to cover the membrane insert. Co-cultures were incubated at 37ºC under a CO_2 atmosphere for various times according to the experiment. After each treatment, incubation medium was aspirated and HCS monolayers were washed twice with PBS to remove any dead cell and kept at -80ºC until processed. For each experiment one rat was used for the isolation and growth of Sc and another one for the isolation of HCS.

LDH measurement

Lactate dehydrogenase (LDH) activity was measured in the culture medium by an enzymatic method (Monotest LDH from Boerhinger-Mannheim Diagnostica/FRG) using a Beckman DU-8 UV-Visible spectrophotometer.

Glutathione measurement

HCS glutathione content was measured by the spectrophotometric recycling method of Tietze (Tietze, 1969) modified by Catala and Portoles (2002), in the presence of 5,5'-ditiobis (2-nitrobenzoic acid) (DTNB), NADPH and glutathione reductase (GR) in a DU70 Beckman spectrophotometer (CA, USA).

Protein content was determined by the method of Lowry et al. (1951).

Nitric oxide measurement

The presence of NO_x species was measured in the culture medium by a NO kit based on De Griess reaction (from Boerhinger-Mannheim Diagnostica/FRG) using a Beckman DU-8 UV-Visible spectrophotometer.

Statistical analysis

Each experiment was performed three independent times with cells isolated from different animals. During each experiment three or four individual culture flaks or wells were assayed. Thus the mean was finally calculated with the values of at least 9 replicates.

Data given in figures and tables represent the mean values ± SD of the mean, taking into account the degrees of freedom lost with each group of replicates.

Comparison between groups has been carried out by the Student's t-test. Results were considered statistically significantly different when $p<0.05$.

Results

Sinusoidal cell characterization

Kupffer cell purification and immunolabelling with ED1-PE monoclonal antibody

The monoclonal antibody ED1 specifically recognizes rat macrophages and can be obtained conjugated to PE commercially. To ensure that all Kupffer cells were stained positively with ED1-PE and that endothelial cells were not stained, pure fractions of Kc and Ec were obtained by centrifugal elutriation at 4ºC. When the interaction of ED1-PE with each cell type was separately evaluated by flow cytometry after treatment for 30 min with the monoclonal antibody, all of the Kc were stained positively (Fig. 2) but the Ec did not stain with ED1-PE. This observation demonstrates the high specifity of immunolabelling with ED1-PE.

Sinusoidal cells in culture, confocal microscopy

In 2-week cultures of sinusoidal cells, 50% of the cells are immunolabelled with ED1-HCS, showing a rounded and compact morphology (Fig. 3) suggestive of being Kupffer cells. In the presence of DCFH-DA all sinusoidal cells emit fluorescence, corresponding to basal levels of free radicals produced in normal metabolism. Endothelial cells present spindle shaped morphology and clearly contain lower levels of free radicals than Kupffer cells (Fig. 4). We can still observe the macrophages showing a compact and rounded shape, with small radial protuberances indicating the emission of short cell processes.

Effect of SC conditioned medium on HCS glutathione content

It has been demonstrated that mediators released by different cell types, e.g. Kc, regulate HCS glutathione content, especially after activation. In the present study this effect has been analysed by treatment of HCS cultures with Sc conditioned medium and the results are shown in Fig. 5. Two different media of cultured Sc in basal conditions for different pre-conditioning periods were used: 15 min and 120 min (referred to as 15'Sc-medium and 120'Sc-medium, respectively). As it can be observed, the metabolites released by Sc are able to reduce significantly HCS glutathione content at short times. However pre-conditioning time is determinant at longer times when 120'Sc-medium has no longer effect while 15'Sc-medium still exerts a significant decreasing action on glutathione.

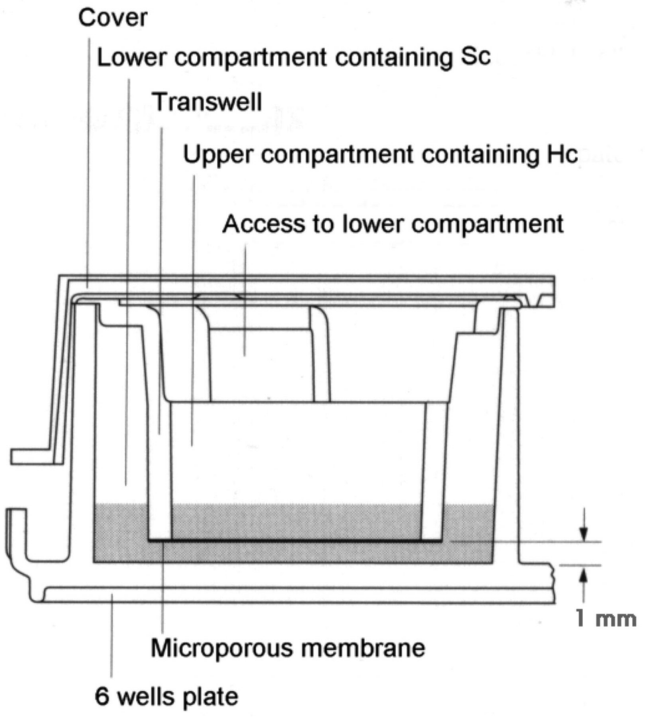

Fig. 1. Schematic representation of co-culture of sinusoidal cells and hepatocytes in Costar Transwells®.

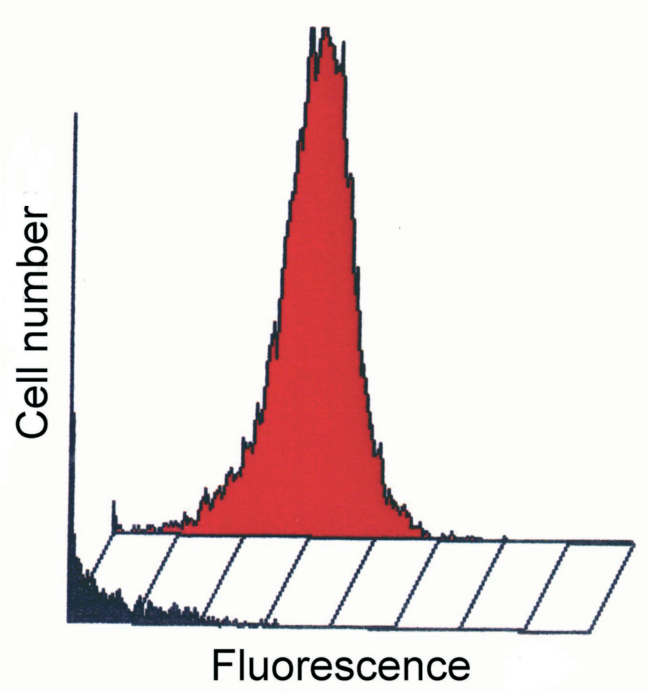

Fig. 2. Flow cytometric characterization of Kupffer cells immunolabelled with the monoclonal antibody ED1 conjugated to PE. The figure represents the number of cells versus fluorescence intensity emitted at 575nm by non labelled (black) and labelled (red) with the monoclonal antibody ED1-PE specific for rat macrophages.

Effect of the co-culture with sinusoidal cells on hepatocyte function

Lactate dehydrogenase release

In order to know the effect of the co-culture of sinusoidal cells with parenchymal cells on the cell membrane integrity, the levels of lactate dehydrogenase (LDH) were evaluated in the culture medium for different times. Figure 6 shows that there is a peak of lactate dehydrogenase release just after the establishment of the co-culture. This effect can be produced by medium replacement stress but at 30 min LDH release is minimal and increases slowly up to 120 min.

Release of NO_X-derived species

Nitric oxide is a small molecule which plays an important role in the communication among liver cells and regulates important liver functions. Ec possess a constitutive nitric oxide synthase (cNOS) while NO synthesis in Kc and HCS is performed by inducible (iNOS) isoforms which are able to synthesize high amounts of NO under certain stimuli. The study of NOx-derived species in the medium of co-cultured sinusoidal and parenchymal cells, Figure 7, shows that after an initial release of NO there is a stabilization of the levels.

Parenchymal cells'glutathione content

Former works have reported the importance of regulation of HCS glutathione content by co-cultured cells in different models. The porous membrane co-culture system resembles anatomical display of Sc and HCS in the liver in which they are very close but physically separated by the space of Disse. As it can be observed in Figure 8, Sc co-culture induces a significant, strong and maintained reduction of HCS glutathione levels during the first 120 min of interaction. Glutathione content of control pure HCS cultures is also

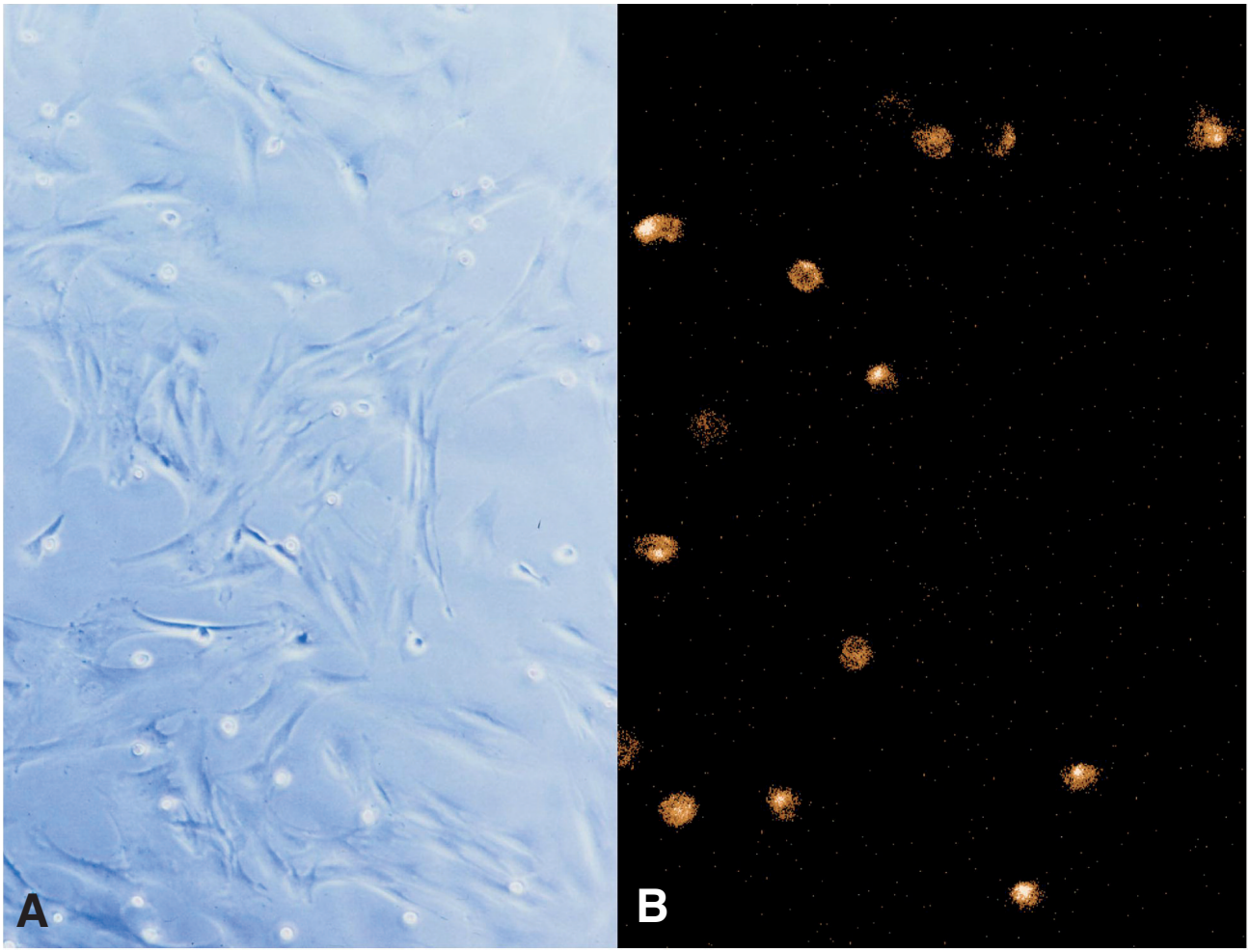

Fig. 3. A. Sinusoidal cell culture enriched in Kupffer cells. Phase contrast. x 1000. **B.** Fluorescence of Kupffer cells immunolabelled with ED1-PE in a sinusoidal cell culture. Confocal microscopy. x 800

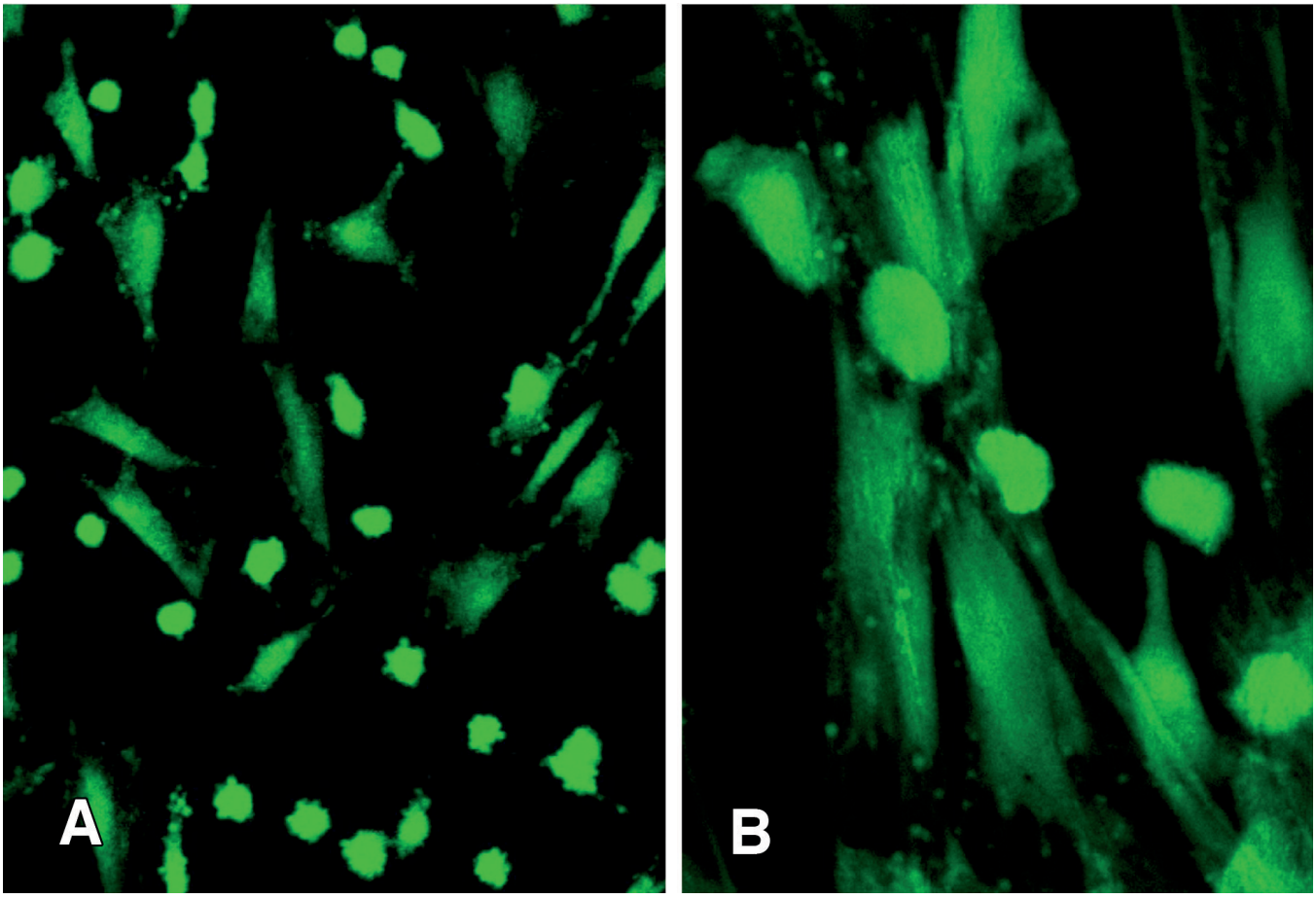

Fig. 4. Free radical content of sinusoidal cells as revealed by the fluorescence emission of DCF. Confocal microscopy. A x 600, B x 1400

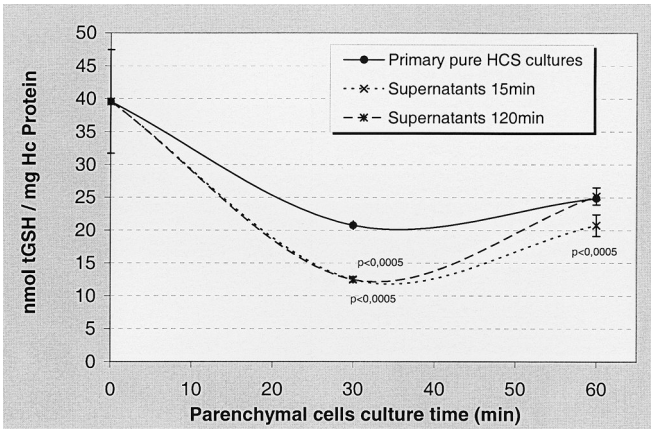

Fig. 5. Effect of sinusoidal cells supernatants incubated 15 or 120 min on cultured parenchymal cells glutathione content. Basal values are represented by the glutathione content of primary pure parenchymal cell cultures.

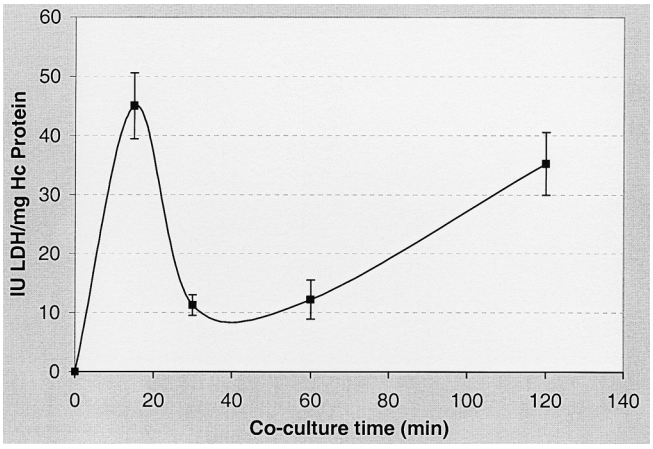

Fig. 6. Determination of LDH in sinusoidal and parenchymal cells co-culture media.

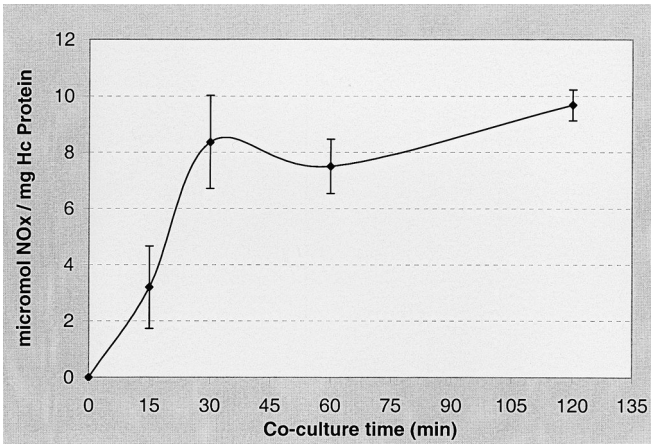

Fig. 7. Determination of NO derived species in supernatants of hepatic sinusoidal and parenchymal cells co-cultures.

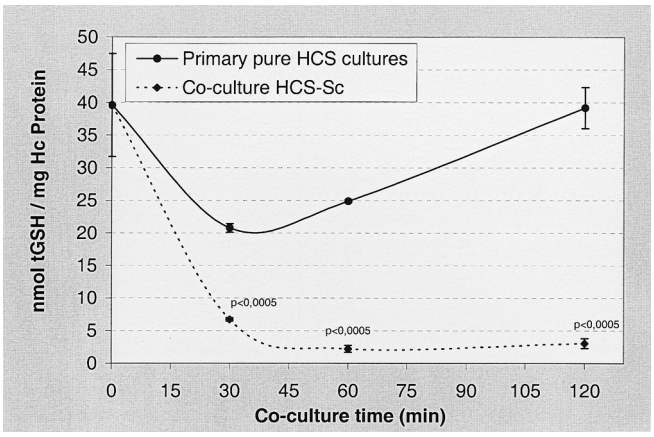

Fig. 8. Parenchymal cell glutathione content in co-culture with sinusoidal cells. Basal values are represented by the glutathione content of primary pure parenchymal cell cultures.

decreased at 30-60 min, probably due to the stress caused by medium change, but levels are completely restored at 120 min.

Discussion

Kc and Ec separation from isolated Sc suspensions is difficult because both cell types have similar sizes and densities. Traditionally, time consuming methods involving functional properties of Kc, such as differential adhesion, have been used. However, centrifugal elutriation is a suitable technique for the rapid and accurate separation of cell types similar in size and density. Besides, morphological differences between Kc and Ec in an established culture are subtle; methods based on functional characteristics are long and sometimes provide only an estimation of each cell type. The use of immunologic markers conjugated to fluorescent probes appears to be a rapid and reliable method for the identification of both cell types by flow cytometry or fluorescence microscopy. The use of immunolabelling and flow cytometry allows a precise characterization of sinusoidal cell populations used for cellular communication studies. Binding of ED1 to Kupffer cells is an evidence of membrane integrity in freshly isolated cells and conservation of differentiation markers in culture. The relative percentage of Kc in isolated sinusoidal cells is 30% (Catala et al., 1999) and we have shown that it increases during culture to 50%. This variation can be due to the known higher adhesion capacity of Kc and agrees with previous reports (Kashiwazaki et al., 1986; Morin and Normand, 1986). Since Bhatnagar et al. (1981) demonstrated that Kupffer cells were able to release superoxide radicals upon activation in vitro, it has been well established the capacity of these cells to produce and secrete different types of reactive oxygen species (ROS) (reviewed in Kmiec, 2001). The use of DCFH-DA and confocal microscopy has allowed us to visualize basal levels of these metabolites both in Kupffer and endothelial cells revealing much higher levels in the macrophages. This intrinsic property could be related to the rapid and potent activation of Kc upon stimulation with LPS demonstrated in vitro (Portoles et al., 1991) and *in vivo* (Bautista et al., 1990; Fukuda et al., 2004).

It has been well established that co-culture of hepatocytes with other epithelial cells, especially from bile ducts, increases and stabilizes drug metabolizing activities such as cytocrhome P450 activity (Begue et al., 1984), glutathione content (Donato et al., 1991; Mertens et al., 1993) and antioxidant enzymes activities (Mertens et al., 1993). There are also an important number of studies reporting that activated Kc strongly modulates HCS functions, causing a decrease in glutathione content (Spitzer and Zhang, 1995) and protein synthesis (Keller et al., 1985; Billiar et al., 1990a; West et al., 1993) or increased apoptosis (Hamada et al., 1999) through the action of different mediators (reviewed in Kmiec, 2001). The paracrine action of HCS mediators on Sc function has also been demonstrated during inflammation (Bankey et al., 1994). However, none of these studies addressed a specific study of Sc effect on HCS glutathione content in basal conditions since they focused on pathological processes. Although the role of cell interactions under normal conditions is far less understood than in pathological ones, it has been demonstrated that Sc enhance HCS protein synthesis and glucose output (Kuiper et al., 1988) and stabilize HCS phenotype in vitro (Morin and Normand, 1986). We report here that the incubation of HCS with Sc soluble mediators produces a reduction of intracellular glutathione which is extremely enhanced if Sc and HCS are maintained in co-culture, which demonstrates a feedback regulation and cellular cross-talk between these cell types. The high levels of ROS in non activated Kc revealed with DCFH-DA arises the possibility of a glutathione decrease mediated by Sc-derived ROS as it was demonstrated for Kc-HCS communication in

endotoxemia (Spitzer and Zhang, 1995). The magnitude of this decrease must be considered with caution given that the strict hepatic cell organization *in vivo* imposes certain restrictions in cell communication (Clemens et al., 1994). In particular they argue that the vascular compartment as well as the perivascular is subject to a convective flow in the periportal-to-perivenous direction and therefore communication via soluble mediators only affects cells downstream.

All three cell types used in the co-cultures are able to express nitric oxide synthases (NOS). Sinusoidal endothelial cells constitutively produce NO through a constitutive NOS (cNOS) to regulate hepatic blood flow. Certain conditions induce the synthesis of high amounts of NO by an inducible, Ca^{2+} independent NOS (iNOS) present in both Sc and HCS. In fact, it has been reported that HCS are the main source of NO in pathological conditions (Clemens, 1999). It seems that regulation of HCS glucose metabolism (Sprangers et al., 1998) and protein synthesis (Curran et al., 1991) by activated Kc is mediated by NO. A series of studies show that NO and glutathione metabolisms are closely related: NO regulates glutathione synthesis (Kuo and Abe, 1996) and glutathione regulates iNOS activity and expression (Duval et al., 1995; Harbrecht et al., 1997), even regulating NO inhibition of glucose metabolism (Padgett and Whorton, 1997). Most of these results have been obtained in activated systems. Our results show that in basal conditions NO production stabilizes shortly after the establishment of Sc-HCS co-cultures which is in agreement with an endothelial constitutive origin of NO. The concordance of the kinetics of NO release to the medium and glutathione decrease in HCS is interesting, and taking into account the former discussion about the connexion between glutathione and NO metabolisms, we consider that it deserves further investigation.

Conclusions

Immunostaining, flow cytometry and confocal microscopy are useful tools in the characterization of complex cell mixtures. These techniques have allowed us to demonstrate that Kc possess higher intracellular ROS basal content than Ec in mixed cultures. The co-culture of hepatic Sc and HCS using porous membranes shows that HCS glutathione content in basal conditions is regulated by a Sc-HCS cross-talk. These results seem to agree with the existence of a paracrine circuit in the management of liver oxidative stress proposed by Cutrin et al., 1998. However, further investigation is necessary in order to clarify the role played by NO and other liver cell types, like biliary duct epithelial cells, in glutathione metabolism.

Acknowledgements. This study was supported by research grant from Fundación MMA (2004-033), coordinated by Dr. J. V. Comas (Pediatric Heart Institute, Hospital Universitario “12 de Octubre”, Madrid, Spain).

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Accepted October 25, 2006