ACTA ABP BIOCHIMICA POLONICA

Vol. 57, No. 2/2010
185–191
on-line at: www.actabp.pl

Regular paper

# Influence of 4-hydroxynonenal and spleen cells on primary hepatocyte culture and a novel liver-derived cell line resembling hepatocyte stem cells★

Ana Cipak[1,#], Suzana Borovic[1,#], Morana Jaganjac[1], Nikolaus Bresgen[2], Iva Kirac[3], Ivana Grbesa[1], Lidija Mrakovcic[1], Marina Cindric[4], Mira Scukanec-Spoljar[4], Koraljka Gall-Troselj[1], Marijana Coric[4], Peter Eckl[2] and Neven Zarkovic[1]✉

[1]Division of Molecular Medicine, Rudjer Boskovic Institute, Zagreb, Croatia; [2]Department of Cell Biology, University of Salzburg, Salzburg, Austria; [3]Department of Surgery, University Hospital Sestre milosrdnice, Zagreb, Croatia; [4]Department of Pathology, Medical Faculty, Clinical Hospital Centre Zagreb, Zagreb, Croatia

**Liver is a unique mammalian organ with a great capacity of regeneration related to its function. After surgical resection or injury, hepatic cells, especially hepatocytes, can proliferate rapidly to repair the damage and to regenerate the structure without affecting the function of the liver. Loss of catalase activity during regeneration indicates that oxidative stress is present in the liver not only in pathological conditions but also as a "physiological" factor during regeneration. As we have shown in our previous work, liver stem cell-like cells treated with 4-hydroxynonenal (HNE), a cytotoxic and growth regulating lipid peroxidation product, recover in the presence of spleen cells. In the current study we characterized this novel cell line as liver-derived progenitor/oval-like cells, (LDP/OCs), i.e. functional liver stem-like cells. We showed that LDP/OC were OV6 positive, with abundant glycogen content in the cytoplasm and expressed α-fetoprotein, albumin, biliverdin reductase and γ-glutamyl transferase. Also, we compared their growth *in vitro* with the growth of cultured primary hepatocytes stressed with HNE and co-cultured with autologous spleen cells. The influence of spleen cells on HNE-treated primary hepatocytes and on LDP/OCs showed that spleen cells support in a similar manner the recovery of both types of liver cells indicating their important role in regeneration. Hence, LDP/OC cells may provide a valuable tool to study cell interactions and the role on HNE in liver regeneration.**

**Keywords:** 4-hydroxynonenal, spleen, hepatocyte, novel-liver derived cell line

**Received:** 13 November, 2009; revised**:** 16 May, 2010; accepted**:** 29 May, 2010; available on-line**:** 31 May, 2010

## INTRODUCTION

Since the ancient myth of Prometheus the liver and its ability to regenerate have engaged human imagination. Today, liver diseases have a strong impact on morbidity and mortality in the world (Bauer *et al.*, 2005). Remarkably, after surgical resection or injury, liver cells can proliferate rapidly to repair the damage and to regenerate the liver (Michalopoulos & DeFrances, 1997). During regeneration, a population of hepatocytes appears devoid of catalase activity in peroxisomes (Okiawa & Novikoff, 1995). This specific population of hepatocytes declines after complete restoration of the liver mass, but their existence indicates that oxidative stress is present not only in pathological conditions but also as a "physiological" factor during regeneration.

When hepatocytes are unable to divide and replace damaged tissues, a subpopulation of liver cells denoted "oval cells" or "hepatic progenitor cells" is triggered to proliferate and regenerate severely damaged liver (Michalopoulos & DeFrances, 1997). Activated oval cells undergo proliferation, migration and differentiation into both hepatocytes and biliary cells (Alison *et al.*, 1998; 2001). In rodents, oval cells also have the lineage potential of uncommitted gastrointestinal stem cells (Alison & Sarraf, 1998). Oval cells share the gene expression profile with other cells resident in the liver. Oval cells are known to express α-fetoprotein (AFP) and γ-glutamyl transpeptidase (GGT) like fetal hepatoblasts, but they also express oval cell antigen 6 (OV6) like biliary epithelial cells. It has been reported that oval cells also share some hematopoietic stem cell markers such as thymus cell antigen 1 (Thy1, CD90), c-kit, and cluster domain 34 (CD34) (Alison *et al.*, 2004). These data strongly suggest that oval cells are precursor cells for a heterogeneous population of cells and could play a crucial role in liver regeneration after various damaging conditions. Possible application of these cells includes their use in transplantation. Yet, models have to be developed to ensure the safety of application in humans. Therefore, several cell lines described as "liver-derived progenitor cells" (Spangoli *et al.*, 1998; Fougère-Deschatrette *et al.*, 2006; Duret *et al.*, 2007) have recently been isolated and described.

Among hepatotoxic conditions oxidative stress and in particular lipid peroxidation play a very important role (Poli *et al.*, 1987). Reactive oxygen species (ROS), generated in excess under stressful conditions, damage bioactive macromolecules, nucleic acids, proteins and lipids, affecting the structure and function of the liver cells. Biologically, one of the most intriguing products of lipid peroxidation is *trans*-4-hydroxy-2-nonenal, HNE, a major bioactive marker of lipid peroxidation (Zarko-

✉e-mail: zarkovic@irb.hr

★Presented at the COST B-35 Work Group 4 Open Workshop "Natural and synthetic antioxidants", September 25–26, 2009, Rzeszów, Poland.

#Both authors contributed equally to this study

**Abbreviations:** ACT, β-actin; AFP, α-fetoprotein; ALB, albumin; BLVRA, biliverdine reductase A; CK, 9 cytokeratin 9; CK, 19 cytokeratin 19; EGF, epidermal growth factor; GGT, γ-glutamyl transpeptidase; HNE, 4-hydroxynonenal; LDP/OC, liver-derived progenitor/oval-like cell; OV6, oval cell antigen 6; Thy1, thymus cell antigen.

vic, 2003) also denoted as a "second messenger of free radicals" (Esterbauer *et al.*, 1991; Zarkovic *et al.*, 1993; 1999). HNE is not only cytotoxic, but also acts as a growth regulating factor (Kreuzer *et al.*, 1998; Zarkovic, 2003). HNE regulates cell proliferation and differentiation by interacting with various signal kinases, such as protein kinase C (PKC), mitogen-activated protein kinases (MAPKs) (Leonarduzzi *et al.*, 2004), and also with enzymes like glutathione-*S*-transferase and glutathione reductase (Awashti *et al.*, 2003). In low concentrations HNE can induce defense mechanisms by activating AP-1 and Nfr2 transcription factors (Uchida *et al.*, 1999; D'Archivio *et al.*, 2008). Taking together all these activities, HNE acts on signal transduction pathways and transcription factors promoting detoxification and cell growth.

In our previous work we have shown that a cell line derived from liver damaged by HNE (Cipak *et al.*, 2005), recovers in the presence of spleen cells, which migrate as inflammatory cells to the damaged liver and affect its regeneration. In this report, the aim was to characterize a cell line derived from liver and to evaluate the effects of exposure to HNE and of spleen cells on the cells of liver origin, which should eventually help to clarify liver regeneration mechanisms.

## MATERIALS AND METHODS

**Cell cultures. LDP/OC cells.** A liver-derived progenitor/oval-like cell line (referred to as LDP/OC) was isolated as described previously (Cipak *et al.*, 2005). Briefly, liver was minced by pushing through a 10 ml syringe and put in a beaker on ice for 5 min to allow large particles to settle. The supernatant was removed using a 22-gauge needle syringe and passed through sterile gauze into test tubes. The tubes were spun at $150 \times g$ for 10 min, the supernatants discarded and the pellets resuspended. All visible clumps were removed using a Pasteur pipette so that the cells were dispersed as a single-cell suspension. The primary culture of liver cells was cultivated in RPMI 1640 medium supplemented with 20% (v/v) fetal calf serum (FCS, Serva, Germany) at 37 °C in a humidified air atmosphere with 5% $CO_2$.

**Primary hepatocyte culture.** Female Fischer rats (strain F 344) weighing approx. 140 g were housed in hanging cages in a temperature (22 °C ± 2 °C) and humidity (65% ± 10% relative humidity) controlled room with a 12 h light-dark cycle. Food and water were provided *ad libitum*. Sawdust served as bedding material. The animals were allowed to acclimatize for at least two weeks prior to hepatocyte isolation.

Hepatocytes were isolated by the *in situ* two-step collagenase perfusion technique as described before (Michalopoulos *et al.*, 1982). Hepatocytes were plated as described before (Eckl *et al.*, 1987) in serum free Minimum Essential Medium (MEM) containing 1.8 mM calcium supplemented with pyruvate (1 mM), aspartate (0.2 mM), serine (0.2 mM) and penicillin (100 U)/streptomycin (100 μg/mL). The cultures were incubated at 37 °C, 5% $CO_2$ and 95% relative humidity. After an incubation period of 3 h, the medium was exchanged with fresh MEM and the cultures were returned to the incubator.

**Spleen cell culture.** Spleen cells used in the experiments originated from the same animal as the liver cells. Spleens were pressed through a sterile mesh which generated single-cell suspension. The spleen cell suspension was stained with Türk stain for leukocytes (Kemika, Croatia), counted and diluted with RPMI to the desired cell density.

**Glycogen staining.** The content of stored glycogen in LDP/OC cells was determined by the Periodic Acid–Schiff with diastase digestion (PAS-de-PAS) technique. Diastase (or α-amylase) acts on glycogen to depolymerize it into smaller sugar units, maltose and glucose, that are washed out of the section. Briefly, cell smears were made on glass slides, air-dried for one hour and subsequently fixed in 4% paraformaldehyde at room temp. for 15 min as described before (Kulkarni & Khanna, 2006). Slides were then stained with the PAS stain. Slides were oxidized with 0.5% periodic acid for 5 min, rinsed with distilled water for 5 min and treated with Schiff's reagent for 15 min. After rinsing in lukewarm tap water for 5 min, slides were counter stained with Mayer's hematoxylin stain and washed in tap water for 5 min. Slides were then dehydrated, covered with cover slip using a synthetic mounting medium and observed under a light microscope. The specificity of the PAS detection for complex sugars, among which is glycogen, was confirmed by a control treatment of the slides with diastase (1 mg/mL) for 15 min before PAS staining.

**Immunogold labeling of OV6.** To determine the presence of OV6 antigen, a marker of oval cells, putative stem cells that differentiate into multiple cell types in the liver (Dollé *et al.*, 2010), immunoelectron microscopy was used. LDP/OC cells were placed in fresh fixative (mixture of glutaraldehyde/paraformaldehyde in cacodylate buffer) overnight at 4 °C. The cells were then washed in cacodylate buffer, postfixed for 1 h at room temp. with 1% osmium tetroxide (Sigma, USA), dehydrated through a graded series of acetones, infiltrated with and embedded in epoxy resin. Ultrathin sections (90 nm) were cut with a diamond knife on a Reichert ultramicrotome. Sections were mounted directly on mesh nickel grids and immunogold labeling was done as described before (Zivkovic *et al.*, 2005). Monoclonal mouse anti-human/rat OV6 antibody (R&D Systems, USA) was used as the primary antibody at a concentration of 10 μg/mL. After labeling the primary antibody with colloidal gold-conjugated rabbit anti-mouse antibody (Dako, Denmark) sections were counterstained with uranyl acetate (Agar, England) and Reynolds lead nitrate solution and analyzed by electron microscopy (Morgagni 268D, Philips).

**Protein isolation and dot-blot analysis of OV6, CD34 and Thy1.** As it has been reported that oval cells express hematopoietic stem cells markers such as Thy1 and CD34, together with oval cell marker OV6, their expression was analyzed in LDP/OC cells by the dot-blot staining using the LSAB (Labeled StreptAvidin-Biotin, Dako, Denmark) method. The cells were washed and $5 \times 10^5$ cells were resuspended in 500 μl lysis buffer (50 mM Tris base, 150 mM sodium chloride, pH 7.5, 1% Triton-X-100, 0.5% Na-deoxycholate, 0.1% SDS, 1 mM PMSF, phenylmethylsulfonyl fluoride). For dot-blot analysis 60 μL of protein extract was spotted onto nitrocellulose membrane (Amersham). The membrane was incubated in blocking solution (1% nonfat milk powder in PBS) at room temp. for 60 min and subsequently incubated overnight with mouse monoclonal antibodies directed against human/rat OV6 (1:40 in 0.01% BSA in PBS; R&DSystems, USA), mouse polyclonal antibodies directed against CD34 (1:100 in 1% BSA in PBS; Santa Cruz, USA), and goat polyclonal antibodies directed against Thy1 (1:100 in 1% BSA in PBS; Santa Cruz, USA). Blots were then washed and incubated with

**Table 1. Primer sequences, annealing temperatures and expected size of amplified products for PC**

| Gene | Primer (forward/reverse) | | Annealing temperature | No. cycles | Product size (bp) |
|---|---|---|---|---|---|
| *ALB* | fw | AAGGCACCCCGATTACTCCG | 61°C | 42 | 649 |
| | rv | TGCGAAGTCACCCATCACCG | | | |
| *AFP* | fw | CAGGAGGAAGAAAGGACAAAAAA | 57°C | 35 | 106 |
| | rv | ATTCCTAAGGCATAGAAATCCCA | | | |
| *BCL2* | fw | TCGCTCTGTGGATGACTGA | 63°C | 38 | 380 |
| | rv | TTTGCCTGAATGTGTGTGTG | | | |
| *BLVRA* | fw | CGTGGAACTCTTGATGGA | 59°C | 38 | 516 |
| | rv | CACTTCTTCTGGTGGCAA | | | |
| *GGT1* | fw | AGAGTTCAGCGGGAGACA | 59°C | 38 | 520 |
| | rv | GGTGCTGTTGTAGATGGTGA | | | |
| *MYC* | fw | CATCTGCGATCCTGACGAT | 59°C | 38 | 450 |
| | rv | GGGTTGCCTCTTTTCCACA | | | |
| *ACT* | fw | ATGTACGTAGCCATCCAGGC | 57°C | 38 | 216 |
| | rv | TGTGGTGGTGAAGCTGTA | | | |

*ALB*, albumin; *AFP*, α-fetoprotein; *BLVRA*, biliverdin reductase A; *GGT1*, γ-glutamyl transpeptidase 1; *ACT*, β-Actin; fw, forward; rv, reverse

Link reagent (LSAB kit, Dako, Denmark) for 30 min, followed by streptavidin-HRP (horseradish peroxidase) (LSAB kit, Dako, Denmark) for another 30 min. Immunocomplexes were visualized using 3,3'-diaminobenzidine tetrahydrocloride (Dako, Denmark) staining and scanned for quantification of signals. Negative controls were included in all experiments in which the antibody tested was omitted and replaced by diluent (1% BSA in PBS).

**RNA extraction and RT-PCR.** Total RNA was extracted from LDP/OC cells using RNA Bee reagent according to the manufacturer's instructions (Biogenesis Ltd., Poole, England) and purified using Qiagen RNeasy® Mini Kit with on-column DNase digestion according to manufacturer's instructions (Qiagen, Valencia, CA, USA). RNA quality, purity, and integrity were verified by absorbance at 260 and 280 nm and agarose (1%) gel electrophoresis.

Purified RNA was converted to cDNA using an oligo$(dT)_{18}$ primer (Roche), 0.5 mM dNTP, 20 U RNase Inhibitor (Roche), 1× incubation buffer (supplied with AMV RT) and 40 U AMV reverse transcriptase (Roche) at 65°C for 10 min and at 42°C for 1 h. The PCR reaction was done in 25 μL reaction mixture containing dNTPs (50 μM each), 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris/HCl (pH 8.3), 6 pmol of each primer (Table 1) and 0.25 U of *Taq* polymerase (TaKaRa). The parameters were 94°C for 2 min, 94°C for 30 s, annealing temperature specific for each primer set (albumin (*ALB*), α-fetoprotein (*AFP*), BCL2, biliverdin reductase a (*BLVRA*), γ-glutamyl transferase (*GGT1*), *MYC* and β-actin (*ACT*) (Table 1)) 72°C for 30 s, for a total number of cycles indicated in Table 1 for each gene. The PCR products were electrophoresed through 1% agarose gel and stained with ethidium bromide (0.5 μg/mL).

**Experimental conditions. LDP/OC cells.** LDP/OC cells were counted and $5 \times 10^5$ cells were incubated in RPMI, 5% FCS with or without 100 μM HNE at 37°C for 30 min. After treatment, liver cells were centrifuged, diluted and plated for the experiments. The spleen cell suspension was counted, diluted to a 1:40 ratio (liver to spleen cell co-cultures) and plated in co-cultures with liver cells. Immediately after plating, and after 24, 48 or 72 h aliquots of 50 μL cell suspension were mixed with 50 μL Trypan Blue, and counted in a Bürker-Türk chamber to determine the viable cell number during experiment. Results were expressed as percentage of the cell count obtained immediately after plating.

**Primary hepatocyte culture.** To determine the percentage of mitotic, apoptotic and necrotic cells by DAPI staining, isolated hepatocytes were incubated with 10 μM HNE for 3 h approx. 17 h after the first exchange of the medium. Thereafter, the medium was replaced by the spleen cell suspension to yield a hepatocyte**:** spleen cell ratio of 1:40, or by plain medium. Epidermal growth factor, EGF (40 ng/mL) and insulin (90 mg/mL) were added to stimulate hepatocyte proliferation. After an incubation period of 48 h cells were washed and fixed with methanol:glacial acetic acid, 3:1 (v/v). After fixation, cells were air dried, washed with McIlvaine buffer, and incubated with DAPI for 60 min. DAPI solution was discarded, and samples were washed with McIlvaine buffer and distilled water and air-dried. Analysis of necrotic, apoptotic and mitotic cells was done under a fluorescence microscope (Leitz Aristoplan). Since exact quantification of apoptotic events is not possible by use of electrophoretic methods (DNA-laddering), and the TUNEL assay fails to discriminate between apoptotic and necrotic cells in hepatocytes (Grasl-Kraupp *et al.*, 1995), the morphology of DAPI stained nuclei was used to unequivocally discriminate between apoptosis (crescent-like condensed or fragmented chromatin) and necrosis (small, pyknotic nuclei revealing highly condensed chromatin) as suggested previously (Oberhammer *et al.*, 1992). This method has a further advantage that the frequency of apoptotic, necrotic and mitotic events as well as micronuclei can be determined in parallel in a single culture.

**Statistical analysis.** All experiments were performed in quadruplicates. Statistical analyses were performed using the Student's *t*-test. Values of $P < 0.05$ were considered significant.

## RESULTS

### Characterization of the liver-derived cell line LDP/OC

The expression profile of the novel LDP/OC cell culture is shown in Table 2. LDP/OC cells were analyzed for gene expression which showed the presence of α-fetoprotein (AFP), albumin (ALB), biliverdin reductase (BLVRA) and γ-glutamyl transferase (GGT1), and also abundant presence of MYC (Fig. 1).

Dot-blot analysis was also used for determination of CD34, Thy1 and OV6. Staining for the CD34 and Thy1

**Table 2. Expression profile of LDP/OC cell culture compared to expression profiles of oval cells, hepatoblasts, and hepatocytes**

| | Oval Cell | Hepatoblast | LDP/OC | Hepatocytes |
|---|---|---|---|---|
| *AFP* | + | + | + | - |
| *ALB* | + | + | + | + |
| *BLVRA* | ? | ? | + | + |
| CD34 | + | + | - | - |
| *GGT1* | + | + | + | - |
| Glycogen | ? | ? | + | + |
| OV6 | + | + | + | - |
| Thy1 | + | + | - | - |

antigens was negative (Fig. 1). The absence of CD34 and Thy1 indicates that the cells could display characteristics of mature hepatocytes, which was confirmed by abundant presence of glycogen in most of the cells (Fig. 1). On the other hand, dot-blot analysis of OV6 showed weak positivity compared to the negative control without primary antibody. For this reason, OV6 was also detected by immunoelectron microscopy (Fig. 2). Immunogold staining of the LDP/OC cells showed a dispersed distribution of the OV6 antigen throughout the cytoplasm.

### Effects of spleen cells on the viability of LDP/OC cells

LDP/OC cells increased the viable cell number in culture during 3 days regardless of spleen cell presence (Fig. 3). Treatment with HNE significantly decreased viable cell number ($P<0.001$) in all time points measured. Interestingly, when LDP/OC cells were treated with HNE and cultivated in the presence of spleen cells, the number of viable LDP/OC cells increased on the 3rd day of experiment. Still, the number of viable LDP/OC cells was significantly lower compared to LDP/OC cells without HNE treatment cultivated with or without spleen cells ($P<0.001$).

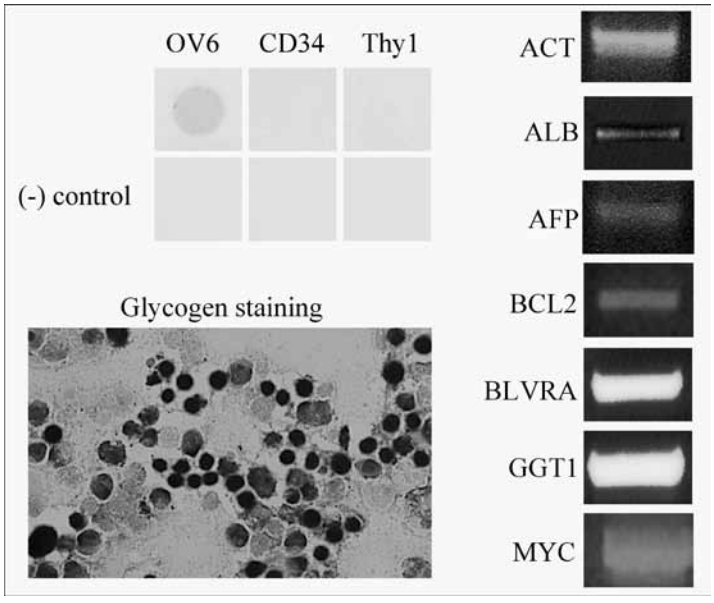


**Figure 1. Expression profile of LDP/OC cells detected by dot-blot, PAS-de-PAS staining and PCR methods.**
Antigens OV6, CD34 and Thy1 were analyzed by dot-blot in comparison with cell samples without primary antibody as negative control. Glycogen content was determined by PAS-de-PAS staining (magnification 200×). Albumin (*ALB*), α-fetoprotein (*AFP*), *BCL2*, biliverdin reductase A (*BLVRA*), γ-glutamyl transferase (*GGT1*) and *MYC* RNA expression was analysed by RT-PCR.

### Effects of spleen cells on apoptosis and necrosis in primary hepatocyte cultures

In order to evaluate the significance of the results obtained with the LDP/OC cells, a primary hepatocyte culture was used. Due to special conditions needed to establish and to maintain the primary hepatocyte culture without losing their characteristics and function, the mitotic index, apoptosis and necrosis were determined for the effects of spleen cells and HNE. For the same reason, HNE was used at a lower concentration, since the cytotoxic concentration used in previous experiment would have caused necrosis. There was no difference in the percentage of mitotic cells between control hepatocytes and hepatocytes treated with HNE cultivated either alone or with spleen cells (Fig. 4). When exposed to HNE, there was a statistically significant increase ($P<0.05$) of apoptosis and also necrosis of primary hepatocytes. Primary hepatocytes showed a significant increase ($P<0.001$) in apoptosis, and also necrosis when cultivated in the presence of spleen cells. In contrast, when hepatocytes were exposed to HNE and cultivated with spleen cells, there was a significant decrease ($P<0.001$) of apoptosis and necrosis compared to hepatocytes exposed to HNE but cultured without spleen cells. These results show similarity to the results for LDP/OC cells published previously (Cipak *et al.*, 2005).

## DISCUSSION

Primary hepatocytes are used in a variety of studies ranging from liver repair mechanisms to liver regeneration processes and in cell transplantation with or without matrix components (Chen *et al.*, 1983; Selden & Hodgson, 2004; Bruzzonea & Strom, 2006; Oertel & Shafritz, 2008). However, there remains a problem of their quiescence and culture stability when the cell culture is to be established. Most common solutions designed to meet such a demand are the use of hepatocellular carcinoma cell lines or immortalization of primary hepatocyte cultures (Anderson *et al.*, 1996; Gondeau *et al.*, 2009). Still, these solutions are not satisfying in studies of complex events including interactions between different types of cells in processes such as liver regeneration. At the end of the development, liver is populated with mesodermal cells originating from hematopoetic cells such as Kupffer cells, natural killer cells and T cells, sinusoidal endothelial cells and bone marrow cells and, lastly, parenchymal cells (Thorgeirsson & Grisham, 2006). The parenchymal cells comprise mature hepatocytes (those producing albumin, biliverdin reductase, glycogen, etc.), biliary cells (expressing CK 9 and CK19), and progenitor cells (denoted oval cells, immature hepatocytes, expressing OV6 and Thy1), which reside near the portal triade in the canals of Hering, and a small fraction of hepatic stem cells, lower than 0.01%. The latter are believed to originate from hematopoetic stem cells in the bone marrow

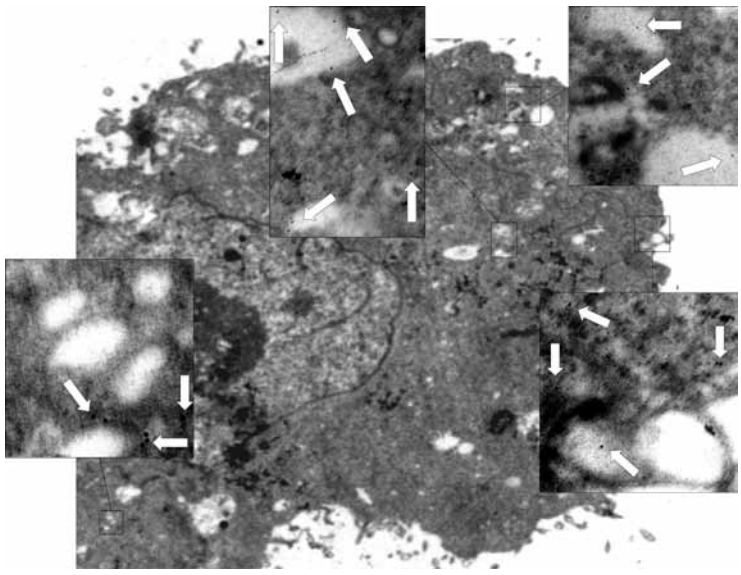

**Figure 2. Detection of OV6 antigen in LDP/OC cells by immunoelectron microscopy (magnification 24500×).**
Mouse anti-OV6 monoclonal antibodies labeled with 10 nm gold particles are indicated by arrows.

(expressing CD34 and c-kit) (Paku *et al.*, 2001; Alison *et al.*, 2004; Qin *et al.*, 2004).

Previously we showed that LDP/OC cells damaged by HNE recovered in the presence of spleen cells (Cipak *et al.*, 2005). Those cells were afterwards successfully used to test novel antioxidative compounds for their possible cytotoxicity and antioxidant capacity *in vitro*, taking into account the potential use of those cells for development of new hepatoprotective compounds (Borovic *et al.*, 2006), assuming that this particular liver-derived cell line shares with primary hepatocytes fundamental biological features in respect to growth and regeneration. Therefore the main purpose of the current study was to define the lineage of the LDP/OC cell line. Gene expression analysis showed the presence of albumin, which is expressed in oval cells, hepatoblasts as well as hepatocytes, confirming liver origin. The presence of GGT1 and AFP indicated that the cells could not be mature since expression of these genes is characteristic of hepatoblasts and oval cells, but not mature hepatocytes. Biliverdin reductase is expressed in mature hepatocytes indicating that the cells display certain characteristics of mature hepatocytes. An abundant presence of MYC was found in an immortalized cell line from normal rat liver probably due to the well-described role of MYC in cell immortalization (Simm *et al.*, 1994; Zindy *et al.*, 1998). The absence of CD34 and Thy1, shown by dot-blot analysis, indicates that the cells could display characteristics of mature hepatocytes, which is confirmed by the abundant presence of glycogen in most of the cells. On the other hand, the presence of OV6 in the cytoplasm indicates that the cells are of oval cell origin. Thus, the results obtained indicate that this particular cell line has characteristics of both immature cells represented by oval cells and hepatoblasts, and mature hepatocytes. As it was previously shown (Cipak *et al.*, 2005), LPD/OC are a heterogenic cell population which occupy a niche in between oval cells and mature hepatocytes, classifying them as liver-derived progenitor/oval-like cells (LDP/OC). This lineage of immortalized cells of liver origin may provide a powerful tool in research of liver regeneration control mechanisms. An important advantage of LDP/OC is their stage of differentiation, as they could be differentiated into two lineages covering the complete liver structure and function. Moreover, if their reactivity to liver-damaging conditions, such as lipid peroxidation, and functional interactions with other cell types would resemble those of functional hepatocytes, the LDP/OC cells could be used as a valuable *in vitro* model of liver regeneration important to study further mechanisms of liver growth control.

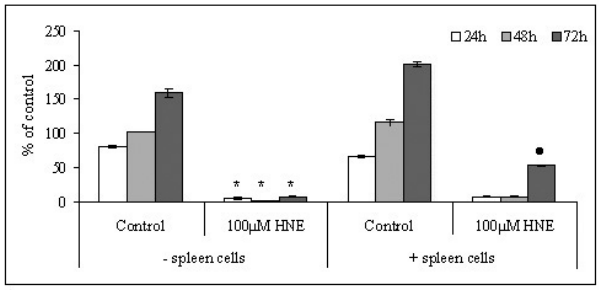


**Figure 3. Effects of spleen cells on LDP/OC cell viability.**
LDP/OCs were untreated or treated with 4-hydroxynonenal (HNE) and cultured either with or without spleen cells. LDP/OC viability was measured with Trypan Blue exclusion assay. *Significantly different compared to untreated hepatocytes; •significantly different compared to HNE treated hepatocytes without spleen cells.

In order to compare the LDP/OC cells with a primary culture of hepatocytes, they were both treated with HNE and mixed with spleen cells. Due to the differences in cell morphology and cultivation conditions different methods were applied to detect the influence of HNE and spleen cells on these cells. Namely, while the growth of primary hepatocytes is anchorage-dependent, LDP/OC cells grow in suspension. The results obtained indicate that spleen cells support the recovery of HNE-damaged liver cells, both primary hepatocytes and LDP/OC cells. As expected, HNE increased the percentage of apoptosis and necrosis in primary hepatocyte cultures, while addition of spleen cells reduced both. As already proposed for LDP/OC cells (Cipak *et al.*, 2005) this beneficial effect of spleen cells could be due to the removal of damaged hepatocytes by spleen cells and/or due to recovery of the damaged hepatocytes supported by spleen cells. Combining the findings obtained for the two different liver cell types it can be assumed that spleen cells migrate to the liver and support the recovery of HNE-damaged liver cells by supporting DNA repair, which can be achieved by various mechanisms relevant to liver regeneration. Secretion of liver growth supporting cytokines, like IL-6, EGF, HGF (Stolz *et al.*, 1999; Streetz *et al.*, 2000; Taub, 2004) by spleen cells is most likely essential, and is supposed to occur at the site of liver damage. Spleen cell migrate to the damaged liver where they exhibit cytotoxic effects on damaged hepatocytes, but without significant effects on the less affected and less differentiated liver cells which contribute to the recovery of the liver mass. When liver cells, either LDP/OC cells or hepatocytes, are oxidatively damaged

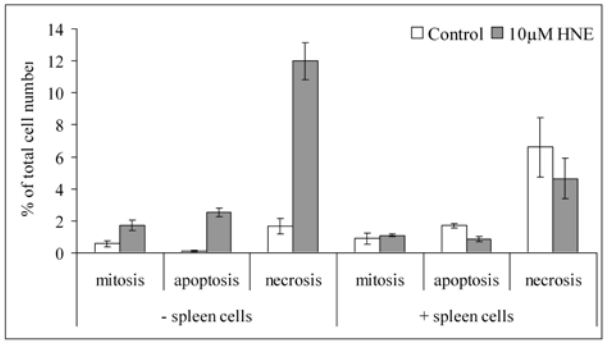


**Figure 4. Effects of spleen cells on primary cultures of hepatocytes.**
Influence of 4-hydroxynonenal treatment and spleen cells on hepatocytes was monitored by DAPI staining and analysis of hepatocyte mitosis, apoptosis and necrosis (*Significantly different compared to untreated hepatocytes; ⁺significantly different compared to hepatocytes cultivated without spleen cells; •significantly different compared to HNE-treated hepatocytes without spleen cells).

with HNE-protein adducts spleen cells appear to support the repair and thus inhibit apoptosis and necrosis of these cells. HNE is known to activate different signal transduction pathways, especially EGF and PDGF receptors (Suc *et al*., 1998; Robino *et al*., 2000), and also the AP-1 and Nfr2 transcription factors (Uchida *et al*., 1999; D'Archivio *et al*., 2008), stimulating its own detoxification. Taking into consideration that there is a loss of catalase activity during liver regeneration and the role of HNE in EGF signal transduction and activation of defense mechanisms, the recovery of HNE-damaged liver cells provides new insight into regeneration mechanisms which should be further investigated.

Finally, it should be pointed out that LDP/OC cells may provide a very useful tool for studying cell interactions during liver regeneration. This study further suggests a new approach in research on the role of oxidative stress in the process of oxidative liver tissue damage and regeneration.

## Acknowledgement

The research was supported by a Croatian-Austrian bilateral project, and Croatian MSES and by COST B35 Action.

## REFERENCES

Alison M, Sarraf C (1998) Hepatic stem cells. *J Hepatol* **29:** 676–682.
Alison M, Golding M, Lalaniel N, Sarraf C (1998) Wound healing in the liver with particular reference to stem cells. *Philos Trans R Soc Lond B Biol Sc* **353:** 877–894.
Alison MR, Poulsom R, Forbes SJ (2001) Update on hepatic stem cells. *Liver* **21:** 367–373.
Alison MR, Vig P, Russo F, Bigger BW, Amofah E, Themis M, Forbes S (2004) Hepatic stem cells**:** from inside and outside the liver? *Cell Prolif* **37:** 1–21.
Anderson K, Yin L, Macdonald C, Grant MH (1996) Immortalized hepatocytes as in vitro model systems for toxicity testing**:** the comparative toxicity of menadione in immortalized cells, primary cultures of hepatocytes and HTC hepatoma cells. *Toxicol In Vitro* **10:** 721–727.
D'Archivio M, Scazzocchio B, Filesi C, Varì R, Maggiorella MT, Sernicola L, Santangelo C, Giovannini C, Masella R (2008) Oxidised LDL up-regulate CD36 expression by the Nrf2 pathway in 3T3-L1 preadipocytes. *FEBS Lett* **582:** 2291–2298.
Awasthi YC, Sharma R, Cheng JZ, Yang Y, Sharma A, Singhal SS, Awasthi S (2003) Role of 4-hydroxynonenal in stress-mediated apoptosis signaling. *Mol Asp Med* **24:** 219–230.
Bauer M, Winning J, Kortgen A (2005) Liver failure. *Curr Opin Anaesthesiol* **18:** 111–116.
Borovic S, Tirzitis G, Tirzite D, Cipak A, Khoschsorur GA, Waeg G, Tatzber F, Scukanec-Spoljar M, Zarkovic N (2006) Bioactive 1,4-dihydroisonicotinic acid derivatives prevent oxidative damage of liver cells. *Eur J Pharm* **537:** 12–19.
Bruzzonea P, Strom SC (2006) Historical aspects of hepatocyte transplantation. *Transplant Proc* 3**8:** 1179–1180.
Chen BP, Berman JJ, Ching WM, Rice JM (1983) DNA breakage by methyl methanesulfonate and its repair in brain and liver term cells cultured from fetal rat and mouse. *Chem Biol Interact* **44:** 63–77.
Cipak A, Borovic S, Scukanec-Spoljar M, Kirac I, Zarkovic N (2005) Possible involvement of 4-hydroxynonenal in splenocyte regulated liver regeneration. *Biofactors* **24:** 217–226.
Dollé L, Best J, Mei J, Al Battah F, Reynaert H, van Grunsven LA, Greets A (2010) The quest for liver progenitor cells: a practical point of view. *J Hepatol* **52:** 117–129.
Duret C, Gerbal-Chaloin S, Ramos J, Fabre JM, Jacquet E, Navarro F, Blanc P, Sa-Cunha A, Maurel P, Daujat-Chavanieu M (2007) Isolation, characterization, and differentiation to hepatocyte-like cells of nonparenchymal epithelial cells from adult human liver. *Stem Cells* **25:** 1779–1790.
Eckl PM, Whitcomb WR, Michalopoulos G, Jirtle RL (1987) Effect of EGF and calcium on adult parenchymal hepatocyte proliferation. *J Cell Physiol* **132:** 363–366.
Esterbauer H, Schaur RJ, Zollner H (1991) Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radic Biol Med* **11:** 81–128.
Fougère-Deschatrette C, Imaizumi-Scherrer T, Strick-Marchand H, Morosan S, Charneau P, Kremsdorf D, Faust DM, Weiss MC (2006) Plasticity of hepatic cell differentiation: bipotential adult mouse liver clonal cell lines competent to differentiate *in vitro* and *in vivo*. *Stem Cells* **24:** 2098–2109.
Gondeau C, Pichard-Garcia L, Maurel P (2009) Cellular models for the screening and development of anti-hepatitis C virus agents. *Pharmacol Therap* **124:** 1–22.
Grasl-Kraupp B, Ruttkay-Nedecky B, Koudelka H, Bukowska K, Bursch W, Schulte-Hermann R (1995) *In situ* detection of fragmented DNA (TUNEL assay) fails to discriminate among apoptosis, necrosis and autolytic cell death**:** a cautionary note. *Hepatology* **21:** 1465–1468.
Kreuzer T, Grube R, Zarkovic N, Schaur RJ (1998) 4-Hydroxynonenal modifies the effects of serum growth factors on the expression of the *c-fos* proto-oncogene and the proliferation of HeLa carcinoma cells. *Free Radic Biol Med* **25:** 42–49.
Kulkarni JS, Khanna A (2006) Functional hepatocyte-like cells derived from mouse embryonic stem cells**:** a novel in vitro hepatotoxicity model for drug screening. *Toxicol In Vitro* **20:** 1014–1022.
Leonarduzzi G, Robbesyn F, Poli G (2004) Signaling kinases modulated by 4-hydroxynonenal. *Free Radic Biol Med* **37:** 1694–1702.
Michalopoulos GK, DeFrances MC (1997) Liver regeneration. *Science* **276:** 60–66.
Michalopoulos G, Cianciulli D, Novotny AR, Kligerman AD, Strom SC, Jirtle RL (1982) Liver regeneration studies with rat hepatocytes in primary culture. *Cancer Res* **42:** 4673–4682.
Oberhammer FA, Pavelka M, Sharma S, Tiefenbacher R, Purchio AF, Bursch W, Schulte-Hermann R (1992) Induction of apoptosis in cultured hepatocytes and in regressing liver by transforming growth factor $\beta_1$. *Proc Natl Acad Sci USA* **89:** 5408–5412.
Oertel M, Shafritz DA (2008) Stem cells, cell transplantation and liver repopulation. *Biochim Biophys Acta* **1782:** 61–74.
Okiawa I, Novikoff PM (1995) Catalase negative peroxisomes**:** transient appearance in rat hepatocytes during liver regeneration after partial hepatectomy. *Am J Path* **146:** 673–686.
Paku S, Schnur J, Nagy P, Thorgeirsson SS (2001) Origin and structural evolution of the early proliferating oval cells in rat liver. *Am J Pathol* **158:** 1313–1323.
Poli G, Albano E, Dianzani MU (1987) The role of lipid peroxidation in liver damage. *Chem Phys Lipids* **45:** 117–142.
Qin AL, Zhou XO, Zhang W, Yu H, Xie Q (2004) Characterization and enrichment of hepatic progenitor cells in adult rat liver. *World J Gastroenterol* **10:** 1480–1486.
Robino G, Parola M, Marra F, Caligiuri A, De Franco RM, Zamara E, Bellomo G, Gentilini P, Pinzani M, Dianzani UM (2000) Interaction between 4-hydroxy-2,3-alkenals and the platelet-derived growth factor-β receptor**:** Reduced tyrosine phosphorylation and downstream signaling in hepatic stellate cells. *J Biol Chem* **275:** 40561–40567.
Selden C, Hodgson H (2004) Cellular therapies for liver replacement. *Transplant Immun* **12:** 273–288.
Simm A, Halle JP, Adam G (1994) Proliferative and metabolic capacity of rat embryo fibroblasts immortalized by c-myc depends on cellular age at oncogenic transfection. *Eur J Cell Biol* **65:** 121–131.

Spagnoli FM, Amicone L, Tripodi M, Weiss MC (1998) Identification of a bipotential precursor cell in hepatic cell lines derived from transgenic mice expressing cyto-Met in the liver. *J Cell Biol* **143:** 1101–1112.

Stolz DB, Mars WM, Petersen BE, Kim TH, Michalopoulos GK (1999) Growth factor signal transduction immediately after two-thirds partial hepatectomy in rat. *Cancer Res* **59:** 3954–3960.

Streetz KL, Luedde T, Manns MP, Trautwein C (2000) Interleukin 6 and liver regeneration. *Gut* **47:** 309–312.

Suc I, Meilhac O, Lajoie-Mazenc I, Vandaele J, Jurgens G, Salvayre R, Nègre-Salvayre A (1998) Activation of EGF receptor by oxidized LDL . *FASEB J* **12:** 665–671.

Taub R (2004) Liver regeneration**:** from myth to mechanism. *Nat Rev Mol Cell Biol* **5:** 836–847.

Thorgeirsson SS, Grisham JW (2006) Hematopoetic cells as hepatocyte stem cells**:** a critical review of the evidence. *Hepatology* **43:** 2–8.

Uchida K, Shiraishi M, Naito Y, Torii Y, Nakamura Y, Osawa T. (1999) Activation of stress signaling pathways by the end product of lipid peroxidation**:** 4-hydroxy-2-nonenal is a potential inducer of intracellular peroxide production. *J Biol Chem* **274:** 2234–2242.

Zarkovic N (2003) 4-Hydroxynonenal as a bioactive marker of pathopysiological processes. *Mol Asp Med* **24:** 281–291.

Zarkovic N, Ilic Z, Jurin M, Scahur RJ, Puhl H, Esterbauer H (1993) Stimulation of HeLa cell growth by physiological concentrations of 4-hydroxynonenal. C*ell Biochem Funct* **11:** 279–286.

Zarkovic N, Zarkovic K, Schaur RJ, Stolc S, Schlag G, Redl H, Waeg G, Borovic S, Loncaric I, Juric G, Hlavka V (1999) 4-Hydroxynonenal as a second messenger of free radicals and growth modifying factor. *Life Sc* **65:** 1901–1904.

Zindy F, Eischen CM, Randle DH, Kamijo T, Cleveland JL, Sherr CJ (1998) Myc signaling *via* the ARF tumor suppressor regulates p53-dependent apoptosis and immortalization. *Genes Dev* **12:** 2424–2433.

Zivkovic M, Zarkovic K, Skrinjar LJ, Waeg G, Poljak-Blazi M, Borovic Sunjic S, Schaur RJ, Zarkovic N (2005) A new method for detection of HNE-histidine conjugates in rat inflammatory cells. *Croat Chem Acta* **78:** 91–98.