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Cytochrome P450 mRNA expressions along with *in vitro* differentiation of hepatocyte precursor cells from fetal, young and old rats

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Abstract: Non-differentiated cells are attractive targets for cell therapy. During liver regeneration oval cells intensively proliferate and differentiate extending their metabolic activity. Hepatic cytochromes P450 (CYPs) can be linked either with metabolic activation of toxic compounds or drug metabolism. We investigated the differentiation and biotransformative potential of non-differentiated cells in primary cell cultures isolated from livers of fetuses (16-days-old), young (4-months-old) and old (20-months-old) rats. Under the conditions of experimental hepatocarcinogenesis, adult rats were fed for three weeks with CDE diet. Liver cells were cultured and precursor cells were differentiated to hepatocytes following induction with sodium butyrate (SB) or dimethyl sulphoxide (DMSO) in culture on MesenCult medium. We identified a number of cells expressing Thy-1, CD34, α-fetoprotein, cytokeratines - CK18 or CK19 and glutathione transferases - GSTπ or GSTα. In vitro differentiation of these cells, isolated from CDE-treated rats begun earlier as compared to non-treated ones. Age-dependent changes in the cell differentiation sequence, as well as CYPmRNA expression sequence accompanying precursor cells differentiation, were also observed. mRNA expression of CYP1A2, CYP2B1/2 and CYP3A1 was higher in the cells of young rats, but in the case of CYP2E1 - in the cells of old rats. It was concluded that both proliferation and differentiation potential of oval cells, decreased with age.

Key words: cytochromes P450, oval cells, cell differentiation, experimental hepatocarcinogenesis, liver regeneration.

Introduction

The population of non-differentiated adult liver cells constitutes 1-3% of normal liver cells, is heterogeneous and consists of stem cells [1,2], and so called oval cells [3] - phenotypically corresponding to fetal hepatoblasts [4,5]. Hepatoblasts are at least bipotential and represent population of progenitor cells either for hepatocytes, and cholangiocytes [6]. It is believed that during liver regeneration and in some pathological conditions oval cells intensively proliferate and differentiate [7]. Both processes are controlled by HGF, TGFα, TGFπ, FGF, IGF-I and IGF-II, modified by influence of mesenchyme, stellate cells, extracellular matrix as well as sex, age, and hepatotoxic agent used in the experimental model due to its individual metabolic activation [8-10]. In consideration of their features oval cells are attractive targets for cell/gene therapy of liver disorders [11].

Among liver cells isolated from rats fed on CDE diet promoting liver injury and regeneration [12], there are oval cells as well as hepatocytes, endothelial cells, fibroblasts and blood cells. Oval cells differentiating to

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Abbreviations: AFP α-fetoprotein; BrdU - bromodeoxyuridin; CDE diet - choline-deficient ethionine-supplemented diet; CK18 and CK19 - cytokeratines CK18 and CK19; DCL(s) - differentiation cell lineage(s); CYP(s) - cytochrome(s) P450; DMSO - dimethyl sulphoxide; GSTα - glutathione S transferase α; GSTπ - glutathione S transferase π; L-PK - pyruvate kinase isoform α; M_2PK - pyruvate kinase; 4m and 20m - 4- and 20-month-old rats, respectively; IOD - integrated optical density; SB - sodium butyrate.

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hepatocytes show expression of developmentally specific markers at different configurations. Initially it is expression of cytokeratines – intermediate filament compounds, as well as fetal α-fetoprotein (AFP) and hepatic albumin [13,14]. In maturating hepatocytes, AFP expression disappear gradually, however, albumin expression increases. Thus, AFP expression is a good indicator of both early developmental lineage of hepatoblasts as well as activated non-differentiated liver cells [15,16]. Between days 3 and 7 of the CDE diet administration some oval cells synthesize fetal form of the pyruvate kinase (M_2PK) and glutathione S transferase π (GSTπ) – the proteins being typical of hepatoblasts of 12 to 13 days-old rat fetus. After 2 weeks of applying CDE diet the oval cells begin to create ductules. Some of the ductular cells express CK19 and GSTπ, the markers specific for cholangiocytic direction of oval cells and hepatoblasts differentiation. Other ductular cells express hepatocyte precursor markers of differentiation appearing between 13 and 15 day (M_2PK and pyruvate kinase isoform α – L-PK), as well as between 16 and 19 day of the rat's development (glutathione transferase α -GSTα). GSTα expression could be detected <4% of parenchymal cells of fetal liver on the 17th day of pregnancy and it grows up during hepatocytes development. GSTπ is absent in hepatocytes of adult rat liver [17]. Therefore, the determinant of hepatocyte differentiation is the appearance of the enzyme protein expression or substitution of fetal enzyme isoform for the mature one, *e.g.* GSTπ→GSTα→GSTμ [6,18]. In turn CK18 appears in bipotential cells and in hepatocyte precursors. Mature hepatocytes, also these being differentiated in culture express both CK8 and CK18 [19,20]. It was shown that in the rats' liver between day 16 and 22 of fetal life, independently from cells possessing the marker CK18, some cells express simultaneously CK18 and Thy -1 [8,15]. Furthermore, oval cells present membrane antigens CD34, Thy -1 and c-kit – common with hemopoietic stem cells [21]. In cultures of fractioned oval and ductular cells, 38% constitute AFP(+), 9% GSTα(+), and over 90% – GSTπ(+) cells. Beyond this, both markers GSTπ and AFP are expressed in some mature parenchymal cells GSTπ(+), particularly in late stages of differentiation [17].

It is also known that hepatoblasts do not express many of the cytochrome P450 isoforms (CYPs), identified in adult mammalian cells. Particular CYPs as well as the mechanism of their expression in fetal rat liver mature in different time in last days of fetal life, which coincides in time with hepatocyte differentiation [22-24]. Hepatic cytochromes P450 from 1, 2 and 3 gene families are the key element of monooxygenase system which can be responsible either for metabolic activation of toxic and carcinogenic compounds or drug metabolism. Thus, the expression of CYP genes in oval cells can have essential implications for the profile of repair processes in regenerating liver and therapy after chemical damage. It may be responsible for both cells exposure to active metabolites and effectiveness of adjunctive therapy.

In this study expression changes of mRNA for CYP1A1, CYP1A2, CYP2B1/2, CYP2E1, CYP3A1 and CYP3A2 accompanying precursor cells differentiation towards hepatocytes were analyzed *in vitro*. To trace CYPmRNA expressions setting in time, hepatoblasts of 16-day-old rat fetuses as well as non-differentiated cells of normal and regenerating liver of adult young and old rats were identified and subjected to differentiation in primary culture on MesenCult medium, applying independently two factors promoting hepatocyte development: sodium butyrate – SB (3.75 mM) or dimethyl sulphoxide DMSO (2%). MesenCult medium is recommended for the enrichment, culture and differentiation of mesenchymal stem cells. Liver regeneration was provoked in model of experimental hepatocarcinogenesis, in which adult rats of different age were fed *ad libitum* on CDE diet for 3 weeks. The presence of non-differentiated cells was confirmed *in vitro* basing on immunohistochemical identification of proliferating cells (bromodeoxyuridin – BrdU – estimation), as well as expressing stem cell (CD34, Thy-1) and oval cell (AFP, GSTα, GSTπ, CK18, CK19) markers.

Material and Methods

Study subject. The study was performed on Sprague-Dawley rats divided into three age groups: 16-days-old fetuses (F16), 4-months-old males (4m) and 20-months-old males (20m). All experiments were approved by the Local Animal Care Committee. Rats were housed at the animal care facility of the Medical University of Silesia. They were subjected to a 12h day/night cycle. Within 4m and 20m age groups, two experimental subgroups were created: one control (C) and one of experimental hepatocarcinogenesis (CDE). Control rats had free access to water and were fed on standard diet. Rats of CDE subgroup were fed *ad libitum* for three weeks on choline-deficient diet supplemented with 0.07% ethionine dissolved in drink water (Altromin, Germany), which injures hepatocytes and stimulates liver regeneration, a process depending on non-differentiated cells.

Analysis of liver morphology. Small pieces of livers taken from rats of all investigated age groups were excised, fixed in formalin, embedded in paraffin and used for the preparation of sections for hematoxyline and eosin (H+E) staining.

Identification of proliferating cells in vivo and in vitro. Proliferating liver cells were identified by detection assay based on Ki67 (Novocastra) and/or bromodeoxyuridin (BrdU; Roche) immunostaining. The latter was based on incorporation of BrdU administered intraperitonally to living adult rats at a concentration of 50 mg/kg per day, every 24 hours for 7 days. Unincorporated BrdU was removed by perfusion of the animals with buffered saline (PBS) prior to sacrificing. Cells, which incorporated BrdU during DNA replication were identified both *in vivo* and *in vitro* using mouse anti-BrdU primary antibody conjugated with peroxidase

Table 1. List of primers used in PCR reactions.

Gene	Sequence of primers	Hybridization temperature (°C)	Product size (bp)
CYP1A1	F) 5'-GAT GCT GAG GAC CAG GAA ACC GC R) 5'-CAG GAG GCT GGA CGA GAA TGC	64.2	679
CYP1A2	F) 5'-CTG CAG AAA ACA GTC CAG GA R) 5'-GAG GGA TGA GAC CAC CGT TG	57.4	139
CYP2B1/2	F) 5'-CCA AGC CGT CCA CGA GAC TT R) 5'-TTG GGA AGC AGG TAC CCT C	56	380/404
CYP2E1	F) 5'-GGA TGT GAC TGA CTG TCT CC R) 5'-TGG GGT AGG TTG GAA GGG AC	54	447
CYP3A1	F) 5'-CCG CCT GGA TTC TGT GCA GA R) 5'-TGG GAG GTG CCT TAT TGG GC	62.9	203
CYP3A2	F) 5'-TTG ATC CGT TGC TCT TGT CA R) 5'-GGC CAG GAA ATA CAA GAC AA	54	323
AFP	F) 5'-CAG TGA GGA GAA ACG GTC CG R) 5'-ATG GTC TGT AGG GCT CGG CC	62.9	252
GAPDH	F) 5'-GTG AAC GGA TTT GGC CGT ATC G R) 5'-ATC ACG CCA CAG CTT TCC AGA GG	66	543

(anti-BrdU-POD) detected by subsequent incubation with substrate (DAB+H_2O_2).

Cell culture. Primary cultures containing non-differentiated cells were sampled from 0.2 g of fetal, or 2 g of normal or regenerating liver. A piece of rat liver was placed immediately in 30 ml MesenCult medium (StemCells, Inc., Canada) designed to the detection, culture and expansion of mesenchymal stem cells, containing glutamine, antibiotics and antimycotics. Subsequently, it was rinsed 3 times for 10 min. with the medium and transferred to sterile Petri dish (φ=100mm), minced (approximately 3 × 3 × 3 mm pieces) and placed in 15 ml sterile conical tube.

The tissue was digested with 20 mg collagenase type II in 10 ml MesenCult, incubated for 60 min. at 37°C (5% CO_2; 95% air), and then decanted. After the supernatant was discarded, 10 ml of trypsin/EDTA (0.05%/0.02%) was added and the sample was incubated for 45 min. to a complete release of cells from the liver tissue. The digestion was terminated by removing the enzyme following centrifugation at 500 × g for 10 min. The remnants of liver tissue were rinsed three times in 10 ml of serum-free MesenCult. All supernatants were polled together and subsequently clarified by centrifugation (10 min; 500 × g; 25°C). The supernatant was discarded and the cell pellet was resuspended in culture flask T75 containing 10 ml MesenCult with 10% fetal bovine serum (FBS) and incubated for 24 hours (37°C, 5% CO^2; 95% air). After that cultures were washed three times with serum-free MesenCult medium; during every rinse the culture incubated for 5 min. under standard culture conditions to remove sticky debris.

Following the last wash, MesenCult medium containing serum was added to the final volume of 10 ml and culture was continued under standard conditions. The medium was changed every two days till culture reached 100% confluence. Subsequently, the cells were lifted up using standard solution of trypsin/EDTA and split in ratio 1:2 for further culture till they reached again 100% confluence.

The cell number was determined as follows: the cells were detached with trypsin (3 ml trypsin/EDTA) and collected by centrifugation (10 min, 500 × g, at 25°C) after stopping the trypsin action by adding 7 ml of MesenCult medium. Supernatant was discarded and cells were resuspended in 1ml of the same medium. Mixture containing 10 μl of cell suspension, 80 μl of the medium and 10 μl of trypane blue was transferred to Bürker's chamber and counted.

The cells were seeded in Chamber Slides (Cover Glass Slide 4 well; LAB-TEK Nalge Nunc Int.), at the density 2×10^4 per well, and incubated (37°C, 5% CO_2, 95% air) 1 day (the cells from young donors) or 2 days (the cells from old rats). Subsequently, BrdU and markers for non-differentiated cells were assayed and differentiating specialty media were added.

Differentiating the cells. To modulate the phenotype of non-differentiated cells in primary culture into hepatocytes, dimethyl sulphoxide – DMSO (2% v/v) or sodium butyrate – SB (3,75 mM) were added to the culture medium during the 28-days lasting experiment. The media were changed for fresh ones every other day. In order to investigate cell differentiation processes, every two days differentiation markers, such as CD34, Thy-1, AFP, CK18, CK19, GSTπ and GSTα were identified using specific antibodies. The differentiation of non-differentiated cells in SB- and DMSO- differentiation cell lineage(s) (DCLs) was performed in duplicate with similar results.

Immunodetection of non-differentiated cell markers. On the particular day for immunostaining, the liver cells cultured in Chamber Slide Cover Glass Slides 4 were washed twice with PBS

Table 2. Number of Ki67(+) cells within 10^4 hepatic cells seen in histological preparation sampled from rats of different age. Data are the mean±SD of measurements done on three different rat liver slices, *p<0.05 (t-Student test).

Age group	Hepatocytes		Non-parenchymalcells	
	Control	CDE	Control	CDE
4m	5±2.3	10±1.0*	15±2.5	51±8.0*
20m		15±1.8*		

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Table 3. Number of cells isolated from fetal and adult rat livers, in primary culture.

Age group and subgroup	Liver sample (g)	Number of cells cultured from 1 g of the liver tissue	Number of cells per chamber	% of all cells in the chamber						
				CD34	Thy-1	AFP	CK18	CK19	GSTπ	GSTα
F16 – C	0.2	3.3×10^6 (on day 3rd)	1×10^4	18	42	65	10	7	15	4
4m – C	2	1.8×10^6 (on day 7th)	2×10^4	2	5	3	11	12	1	2
20m - C	2	0.86×10^6 (on day 14th)	2×10^4	1	5	2	3	3	2	2
4m - CDE	2	1.7×10^6 (on day 7th)	2×10^4	4	16	10	10	19	13	10
20m - CDE	2	0.6×10^6 (on day 14th)	2×10^4	17	24	4	9	14	12	8

(5 min.), and fixed for 15-20 min. using 4% paraformaldehyde. After the removal of fixation solution, slides were washed with PBS. Subsequently, fixed cells were treated with 1% Triton 100 in PBS for 60 min., washed twice with PBS and blocked 60 min. with 1% BSA in PBS. Blocking solution was removed and a primary antibody (1:100) against particular cell marker: Thy-1.1, CD34, CK18, CK19 (Chemicon), GSTπ and GSTα (Novocastra) was added. Antibodies were applied separately and incubated overnight at 4°C. Subsequently, the specimens were rinsed 3 times with PBS and incubated for 1 h with secondary antibodies (1:100) conjugated with alkaline phosphatase or horseradish peroxidase. Reaction was developed and visualized with appropriate BCIP/NBT or DAB liquid substrate system (Sigma), respectively, according to manufacturer recommendations. Primary antibody against AFP (Santa Cruz Biotechnology) was labeled with fluorescent marker rhodamine. In order to visualize nuclei preparations were stained with methyl green and covered with appropriate medium.

To visualize and analyze immunostainings the optical microscope Eclipse E600 (Nikon) fitted with digital camera SSC-DC58AP (Sony) and the confocal fluorescent microscope (Olympus Fluoview IX 70) were used. Total number of cells and a number of positively marked cells were assayed in every culture chamber and expressed as a percent of positively stained cells in total population.

CYP and AFP mRNA expressions. Fetal rat liver RNA was prepared from three individual livers. Total cellular RNA was isolated from 30 mg of fetal rat liver tissue by acid guanidinium thiocyanate/phenol/chloroform extraction [25] using commercially available kit (Trizol TM). RNA was reverse transcribed into cDNA. RT reaction was carried out as previously described [23].

RNA from cultured cells was isolated with RNeasy Mini Kit (Qiagen). The purification procedure was exactly as recommended by the kit supplier. The purified mRNA was used to assay the expression of P450 and AFP following RT-PCR. RT reaction was carried out for 1h at 42°C with the use of oligo-dT as a primer. Subsequently the PCR reaction mixture contained primers specific for rat AFP and CYP1A1, CYP1A2, CYP2B1/B2, CYP2E1, CYP3A1, CYP3A2, and GAPDH was done (Table 1). Amplification was carried out for 30 cycles as follows: denaturation at 94°C for 1 min., then annealing in temperature gradient (54 to 66°C) depending on primer Tm, for 1.5 min. and elongation at 72°C for 1 min. using Programmable Thermal Controller PTC-200 (MJ Research, Inc., Watertown, MA). An aliquot of each reaction mixture was subjected to electrophoresis on 2% agarose gels.

To standardize the protocol the products of GAPDH amplification were always run on the same gel as assayed mRNAs. The gels were stained with ethidium bromide and quantified by densitometry with One D-scan software (Scanalytics).

Results

Liver injury and cell proliferation

In livers sampled from rats fed with CDE diet, degenerative changes within the parenchymal cells and changes of liver acinus organization were found. The pathological changes were more advanced in old animals' livers than in young ones (Fig. 1). Liver injury was accompanied by increased proliferative activity of both, parenchymal and non-parenchymal cells (Fig. 1C-E; Table 2). A positive staining for BrdU detected in nuclei of some liver cells in both C and CDE group, indicated that the cells were dividing *in situ*. BrdU(+) cells were subsequently identified in primary cultures (from 19 to 54% of liver derived cells) confirming that cells proliferating in culture were of the liver origin (Fig. 1E, 1F).

In vitro identification of markers specific to stem cells and oval cells

A quantitative analysis of percentile composition of the primary cultures established from fetal and adult rat livers revealed that all the populations – either control or from CDE group – contained non-differentiated cells positive for stem-like cells markers CD34 or Thy-1 and for oval cells markers: AFP, CK18, CK19, GSTπ, or GSTα (Fig. 2; Table 3).

In primary cultures of fetal cells the percentage of Thy-1(+) cells reached 42% and doubled the number of CD34(+) cells (Table 3). Few times higher number of Thy-1(+) cells than CD34(+) cells was also found in

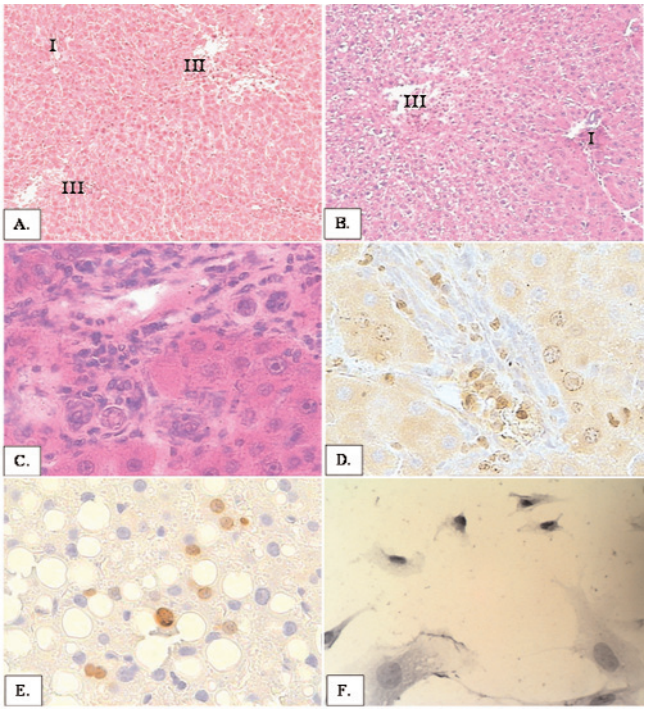

Fig. 1. Morphological changes in livers sampled from adult young (**A**) and old rats (**B**) fed with CDE diet (H+E staining, 100×) and proliferative activity of hepatic cells in old rat liver injured by CDE diet: (**C**) – H+E staining; (**D**) – Ki67(+) cells; (**E**) – BrdU(+) cells; and (**F**) – BrdU(+) cells in primary culture. C-E – 400×; F – 200×; I and III – zone 1 and 3 of the liver acinus, respectively.

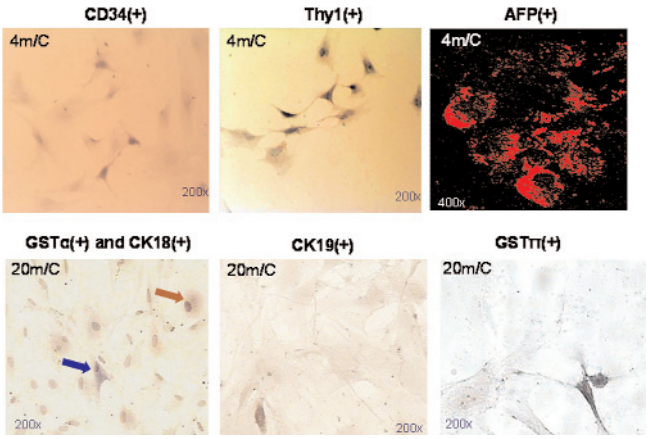

Fig. 2. Immunodetection of non-differentiated cell markers in the primary culture of cells isolated from the liver of adult young (4 m/C) and old (20 m/C) control (C) rats. Blue and brown arrow – GSTα(+) and CK18(+) cells, respectively.

primary cultures obtained from adult rat livers of all subgroups. Moreover, an increase in the content of cells positive for both CD34 and Thy-1 was observed in CDE subgroups as compared to controls. It was significantly higher for cells derived from old livers. For CD34(+) cells its value was close to the cell numbers obtained for cultures established from fetal livers.

In the primary culture of fetal cells, about 65% of them were positive for the AFP. The percentage of AFP(+) cells in cultures established from livers of young and old control rats was very low in controls, however it was 2 (old rats) to 3 (young rats) fold elevated in cultures of cells derived from rats fed on CDE diet (Table 3).

The percentage of the cells with oval cell markers in the total population of cultured cells isolated from adult young and old rats was a few-times elevated in CDE subgroups as compared with controls. This percentage counted for CDE subgroups was comparable (CK18 and GSTπ) or twice elevated (CK19 and GSTα) the corresponding values obtained for cultured fetal cells. Moreover it was 1 to 5% higher in the population of cells obtained from livers of young rats fed on CDE diet when compared to cells isolated from livers of old, CDE-treated rats (Table 3).

In vitro differentiation of fetal liver cells and oval cells

During differentiation, AFP(+) cells were identified by immunofluorescence and AFPmRNA expressions accompanied the number of these cells are presented (Fig. 3).

In both differentiation cell lineages of fetal cells – SB and DMSO – AFPmRNA levels reached their maxima on day 10 and 18 of differentiation, respectively, and subsequently they all decreased (Fig. 3). In general, the levels of AFPmRNA in cells isolated from adult rat livers of CDE subgroups were much higher as compared to adult control animals. The levels of AFPmRNA expression in cells of young rats were higher as compared to old ones, both control and CDE. They were also much higher in MS-DCLs as compared with DMSO-DCLs. Age-dependent time profiles of AFPmRNA expression levels in both DCLs of adult cell cultures were different. In the SB-DCLs of adult rat cells, AFPmRNA content increased gradually to the end of differentiation. In DMSO-DCLs of control cells, corresponding changes in the AFPmRNA expression level occurred earlier during differentiation.

The non-differentiated cells derived from fetuses and young rats were more sensitive to differentiation factors than cells obtained from old ones. In the case of fetal cells the higher increase of cells positive for GSTπ and GSTα was observed in SB-DCLs (Fig. 4A). However, in both DCLs already after day 4 of differentiation GSTα(+) cells entered plateau of growth phase. In SB-DCL of adult rat cells (Fig. 5A) the percentage of GSTπ(+) and GSTα(+) cells raised faster to the maximum value in 4m group (10 to 12 day) than in 20m group (18 to 22 day). At this time the number of

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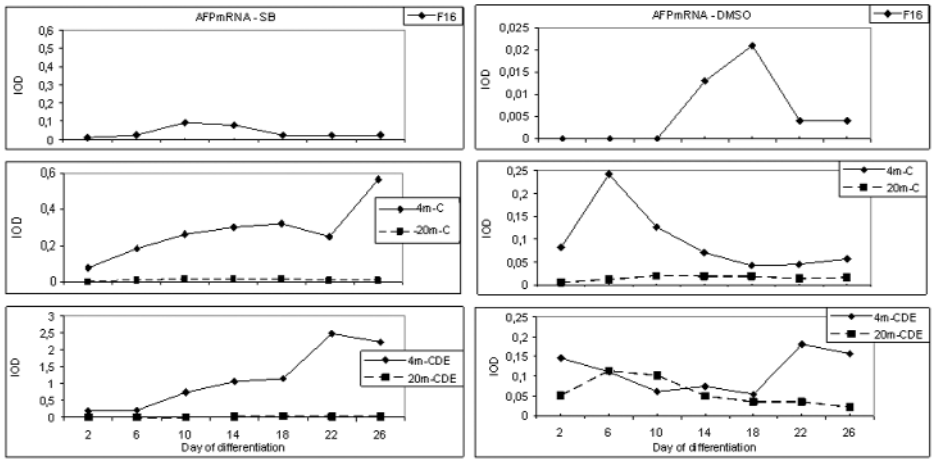

Fig. 3. AFPmRNA expression in fetal and adult cell cultures maintained in MesenCult medium containing SB (**A**) or DMSO (**B**). C – Control; CDE – CDE diet; 4m and 20m: 4- and 20-month-old rats.

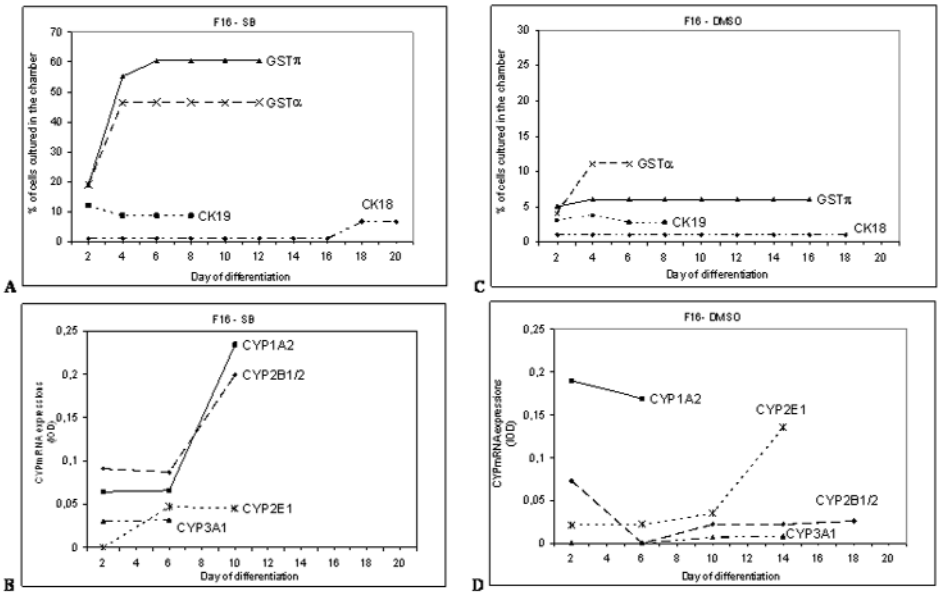

Fig. 4. Increasing percentage of cells positive for oval cell markers (**A** and **C**) and cytochrome P450mRNA expressions (**B** and **D**) in fetal liver cell cultures maintained in Mesen Cult medium containing SB (**A** and **B**) or DMSO (**C** and **D**). IOD – integrated optical density.

GSTα(+) cells derived from young rats was almost 2-fold higher as compared with old rats. An increase of the number of GSTα(+) cells from young rats was accompanied by low increase in CK18(+), but not of CK19(+) cell number. The comparison of SB- and DMSO-DCLs revealed that the changes in corresponding cell number were faster in the later, both in DCLs of cells derived from young rats as well as from the old ones (Fig. 5B). Moreover, earlier, already after day 4 of differentiation the number of CK19(+) cells started to decrease and in day 8 the number of GSTα(+) cells reached a stable level of 15%. In cultures of cells obtained from old rat livers a high number of CK19(+) cells was observed until day 10 but the high number of GSTα(+) and CK18(+) cells was reached faster (in day 4) and, also, GSTα marker disappeared faster. In both DCLs the most stable marker of differentiating oval cells seemed to be GSTπ.

In the SB-DCL of cells derived from young and old donors fed on CDE diet the quantitative changes were earlier than in controls (Fig. 5A); we observed a time shift for maximal number of GSTα(+) and GSTπ(+) cells, to days 2/4 and 4/10, respectively. In the culture from young rats a significant increase in percentage of CK19(+) cells was also observed. In the DMSO-DCL of young donors cells, GSTπ(+), GSTα(+) and CK18(+) cells revealed in higher number than in control already in the second day of differentiation (Fig. 5B). GSTπ(+) and GSTα(+) cells derived from old rats reached the maximal number at day 4 and 6, which was comparable to controls. It was also characteristic that both in DMSO- and in SB-DCL the number of cells with hepatocyte markers decreased faster than in controls.

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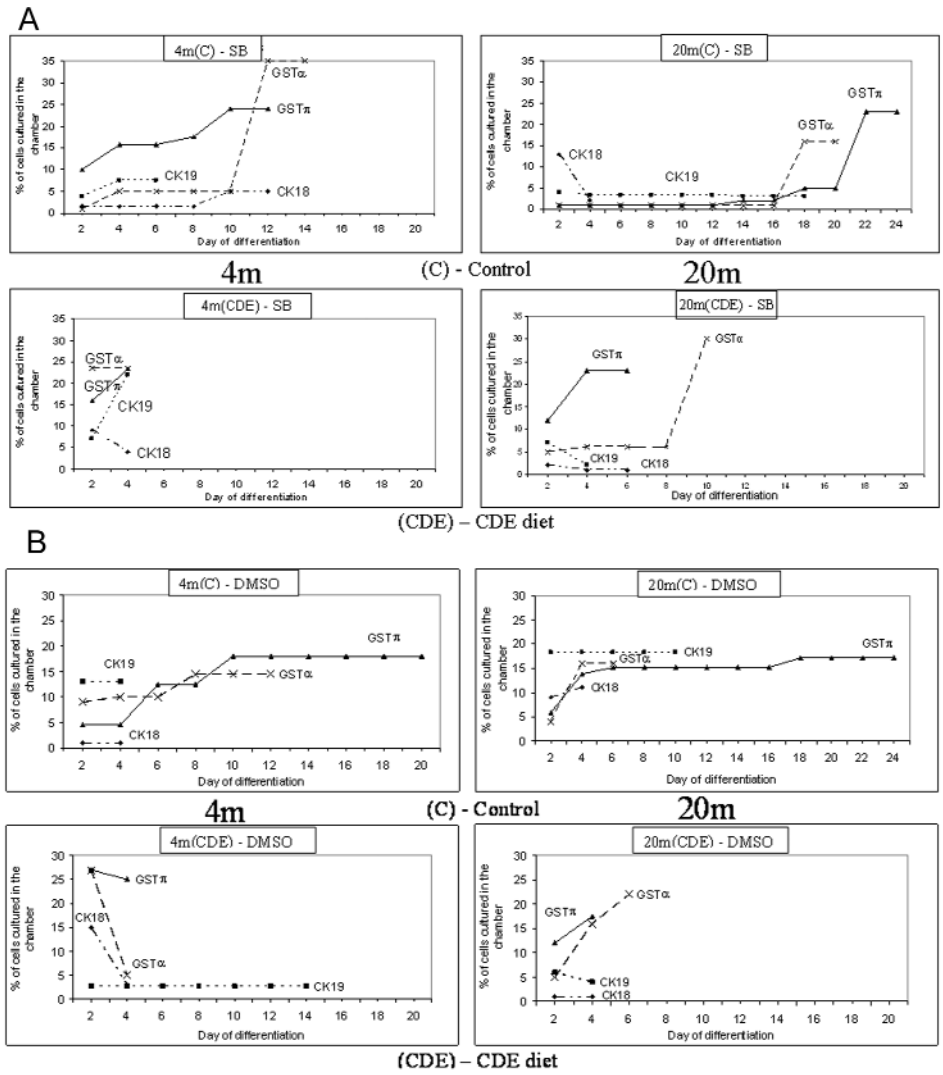

Fig. 5. Percentage of CK18(+), CK19(+), GSTπ(+) and GSTα(+) cells in adult rat liver cultures maintained in MesenCult medium containing SB (**A**) or DMSO (**B**). In most cases descending values are not shown.

Expression of P450 mRNAs in fetal liver and in fetal and adult hepatocyte-precursor cells differentiating in vitro

In the livers of 16-day old fetuses the expression of CYP1A2, CYP2B1/2, CYP2E1, CYP3A1, and CYP3A2 mRNAs was detected (Fig. 6) however, CYP3A2mRNA was not detectable in cells isolated from fetal, and adult donors from both C and CDE subgroups. On the other hand, expression of CYP1A1mRNA was not detectable in fetal livers. It could be found sporadically in SB-DCL were it appeared only at the beginning of the differentiation of fetal cells and cells obtained from adult rats subjected to experimental hepatocarcinogenesis, and it quickly vanished (not shown).

In SB- (Fig. 4B) and DMSO-DCLs (Fig. 4D) of fetal cells CYP1A2mRNA expression was observed from day two of differentiation. At this time it was almost 4-fold lower in SB-DCL than in DMSO-DCL. The expression level in SB-DCL increased 5-fold by day 10 and on day 14 of differentiation it lowered to the starting level. In DMSO-DCL CYP1A2mRNA expression decreased from day 2 to day 10 by about 40%.

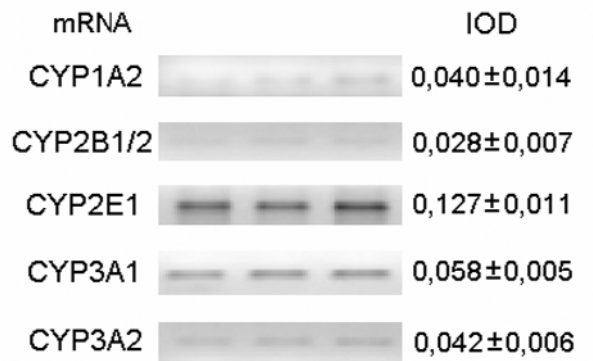

Fig. 6. Constitutive expression of hepatic cytochrome P450 mRNAs in 16-days-old rat fetuses. Rat liver RNA was prepared from three individual livers. Gels obtained from RT-PCR were quantified by densitometry (mean±SD). IOD – integrated optical density.

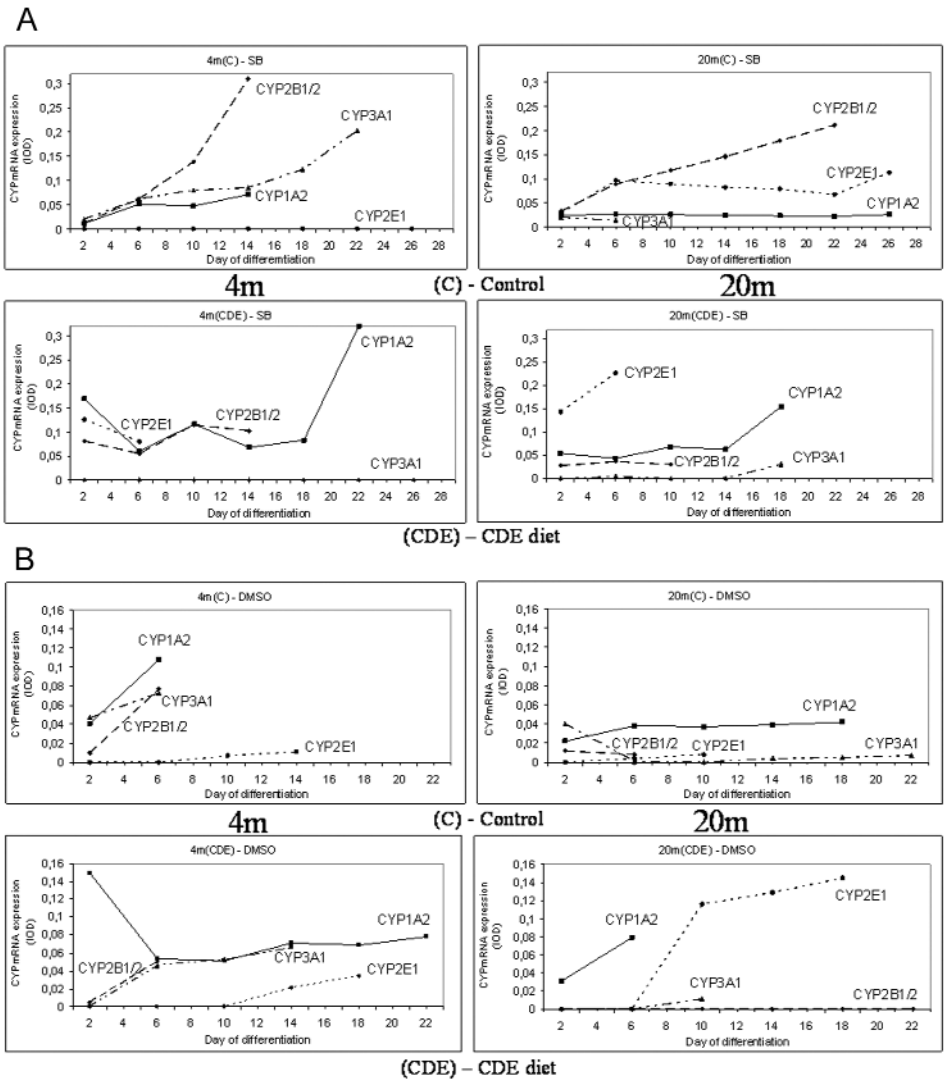

Fig. 7. Increasing mRNA expression of CYP1A2, CYP2B1/2, CYP3A1 and CYP2E1 in liver cells isolated from 4- and 20-months-old rats, maintained in MesenCult medium containing SB (**A**) or DMSO (**B**). IOD – integrated optical density.

In the case of adult control cells expression of CYP1A2mRNA in both SB- (Fig. 7A) and DMSO-DCLs (Fig. 7B) increased faster and reached higher values in cells of young rats as compared with old ones, *e.g.* it increased 7-fold between days 2 and 14. In differentiating cells of old rats this expression was more stable during differentiation. Age-dependence indicating more intense expression of CYP1A2mRNA in differentiating cells of young rats was also found in CDE sub-groups, but there it was at higher levels than in controls.

CYP2B1/2mRNA expression level detected in SB-DCLs of fetal cells between days 2 and 6 of differentiation (Fig. 4B) increased 2-fold at day 10, and then it gradually decreased until day 22. In DMSO-DCLs (Fig. 4D) the expression of CYP2B1/2mRNA disappeared at day 6, however in subsequent days it gradually increased reaching on day 22 half of the initial value. In SB-DCLs of cells from young and old rats CYP2B1/2mRNA appeared relatively fast – from day 6 (Fig. 7A) and the significant progress of expression correlated with time of differentiation. However, in cells of young rats the expression reached the maximum 8 days earlier (at 14 day) and at higher level than in cells from old donors. In cells isolated from animals treated with CDE (both young and old) the expression level for CYP2B1/2mRNA did not change significantly during differentiation but the levels were still higher in cells isolated from the young rats. Similar age-dependence was observed in DMSO-DCLs (Fig. 7B).

In SB-DCLs of fetal cells (Fig. 4B) CYP2E1mRNA expression appeared as early as on day 6 but it was not detectable after day 10 of the differentiation. In DMSO-DCLs (Fig. 4D) the expression was detectable from day 2 of differentiation. It increased 7-fold by day 14 and then – between days 14 and 26 – it decreased by half of the maximal value. The comparison of CYP2E1mRNA expression in SB- and DMSO-DCLs of cells from young and old donors, control and fed on CDE diet indicates (Fig. 7A and 7B)

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that in differentiating cells from old rats it was a few times higher, it appeared faster (*e.g.* in DMSO-DCL – 4 days earlier) or it disappeared later (*e.g.* in SB-DCL – 4 days later).

In SB-DCL of fetal cells the expression of CYP3A1mRNA (detectable until day 6) completely disappeared at day 10 of differentiation (Fig. 4B). In DMSO-DCL the expression started after 6 days of differentiation and vanished after day 14 (Fig. 4D). In differentiating cells from control adult young rats, expression of CYP3A1mRNA appeared fast and reached high levels in both SB-DCL (until 22 days of culture) and DMSO-DCL (until 6 days of culture) (Fig. 7A and 7B). In cells from young rats fed on CDE diet and differentiated in the presence of SB, the expression of CYP3A1mRNA was low or undetectable, however, when differentiated with DMSO the expression levels were comparable to controls. In the cells from old donors the expression was weak and disappeared fast or appeared late.

Discussion

The questions about the origin of oval cells in the context of relationship between stem cell differentiation and dedifferentiation of parenchymal cells, are still open [26-28]. It is known that low doses of ethionine in the CDE diet in first weeks of feeding stimulate proliferation of oval cells without causing necrosis or inflammation [29]. In this study we observed the liver injury caused by CDE diet, namely degenerative changes within the hepatocytes and changes of liver acinus organization accompanied by proliferative activity of liver cells. These changes were more advanced in old animals' livers than in young ones. We confirmed that the liver regeneration could be dependent at least in part on stem cells and oval cells, because in damaged liver increased the number of cells presenting stem cell markers CD34 and Thy-1 and cells resembling immunohistochemically characteristics of oval cells and fetal hepatoblasts, namely presenting markers: AFP, GSTα, GSTπ, and CK18. Moreover, we showed that not only the liver regenerating after CDE diet application and also the normal adult rat's liver may be the source of non-differentiated cells, which enabled an effective isolation of these cells and performance of further studies on their differentiation *in vitro*.

It is unclear whether a number increase of CD34(+) and Thy-1(+) cells observed by us, was exclusively the result of their proliferation or also their enlarged inflow into the damaged liver. More intensive proliferation in old rats' livers which showed the higher degree of damage in comparison to livers of young animals was not equivalent to the enlargement of a number of cells in primary culture. The proof is a different time of duration of culture necessary to breed from taken liver sections a comparable number of cells: about half shorter in case of cells of young individuals in relation to the old ones. Moreover, the cells isolated from livers of rats treated with CDE diet needed so much time themselves to attach to the plate in a comparable number, which the cells of control rats, despite that oval cells of CDE group had in composition of population larger proportional part. It may suggest that despite enlarged proliferating activity *in vivo*, non-differentiated cells isolated from livers of old rats treated with CDE show both smaller viability and proliferative activity *in vitro* (in larger degree oval cells, in smaller degree – stem cells).

We proved previously the ability of liver-derived precursor cells to differentiate toward cell lineages different from those of hepatic origin, *e.g.* neurons, glial cells, osteoblasts and adipocytes [30]. Here we subjected non-differentiated cells isolated from fetal, mature intact and damaged liver to differentiation *in vitro*, independently for help two factors promoting hepatocyte development: SB or DMSO. Application of two promoting factors, limited the mistake in interpretation of results, with reason of incomplete differentiation programme which the cells realized under culture conditions. It was noticed previously that SB added to culture braked the proliferation and stimulated an increase of the size of cell through induction or slowing down the expression of definite genes [31]. Part of the programme of hepatocyte-precursor cell differentiation was the controlling an albumin production, increasing activity of tyrosine aminotransferase (TAT) as well as repression of AFP and DNA synthesis [32]. DMSO stimulated in primary cultures the differentiation of fetal hepatoblasts and adult precursor cells in direction of hepatocytes, and it made possible long-lasting culture of differentiated parenchymal cells – up to 7 months. On the other hand it broke the proliferation of nonparenchymal cells [19]. After the use of both SB and DMSO, mature cells showed the characteristic biochemical features of hepatocytes, *e.g.* albumin expression and increased TAT and G6P-ase activity, as well as lowering γ-glutamyl transferase activity in cholangiocytes. Some features of mature hepatocytes underwent re-expression, for example 35% to 40% cells which differentiated in the culture and lost albumin expression after six passages began its production again, showing the internal plasticity of oval cells [29]. However, parenchymal cells differentiated in culture not always showed typical hepatocytic morphology and function, *e.g.* they were not able to create colonies and to synthesize or couple bile acids [32]. This suggested a deficiency of some components and/or limited possibilities of differentiating the cells under *in vitro* conditions.

In this study SB and DMSO caused the percentage changes in a number of cells exposing particular oval

cell markers in time of duration of 28-days cell cultures. The difference between maturing cells of young and old rats in both differentiating lineages – SB and DMSO – appeared, both in relation to a number of cells expressing given marker and the time in which the number of positively stained cells achieved maximum values. About hepatocytary direction of differentiation indicated increase in AFPmRNA expression level and in a number of AFP(+), GSTπ(+) and GSTα(+) cells, as well as the fall of number of CK19(+) cells in the following days of culture. The potential of differentiation of oval cells taken from young rats, both control and treated with CDE diet seemed higher in comparison with old ones. The results testify that in cultures of stem and oval cells isolated from livers of rats treated with CDE diet, the larger number of oval cells approached to differentiation in direction to hepatocytes in shorter time than the cells from control livers, however, the markers of differentiation disappear more quickly in these cultures as well. A quick loss of phenotype in both lineages of differentiation can indicate distant toxic effects of the CDE diet.

It was observed in different *in vivo* experimental models that oval cells show the deficiency of drug metabolizing enzymes, by which they are resistant to carcinogens. Drawing on investigations with use of 2-AAF, it was reported that oval cells, cholangiocytes as well as hepatocytes stimulated by carcinogen show weak expression of enzymes of phase I and strong expression of phase II drug metabolizing enzymes [33]. In hyperplasic nodules the repression of synthesis of P450 proteins and loss of their catalytic activity, as well as disappearance of CYP1A1 inducibility after 3-methylcholanthrene, took place [34]. In the model of experimental hepatocarcinogenesis with the use of retrorsine, hepatocyte-like precursor cells proliferating in early stages of liver regeneration showed neither mRNA nor protein expression of CYP1A2, CYP2E1 and CYP3A1, both induced in rat liver exposed to this alkaloid [35]. Because cytochromes P450 seem to be essential for metabolizing retrorsine to toxic metabolites, the resistance of progenitor cells to the mitoinhibitory effect of the alkaloid was explained by low levels of P450 expression. In freshly isolated oval cells, isolated from rats fed on CDE diet for 6 weeks and in OC/CDE6 oval cells, cytochrome P450, as well as aminopyrine N-demethylase and ethoxyresorufin-O-deethylase activity was not detected [36].

It seems that oval cells activate the mechanisms of cytochrome P450 expression during differentiation and forming small focuses of basophilic hepatocytes. Golding [33] proved, that the part of ductular cells occurred CYP1A2, CYP2E1 and CYP3A1 expression. In other studies the lack of CYP2E1 and 3A1 expression in progenitor cells as well as the appearance of CYP2B1 expression was similar in chronology to events in fetal period, which underlined the transitory character of non-differentiated cells in the regenerating organ. It was supposed that in control of P450 genes in progenitor cells participate transcriptional factors, such as HNF-1, HNF-3, HNF-4 and C/EBP which were detected in these cells at mRNA level [37].

Our observation of different courses of formation of CYP1A1mRNA (extrahepatic isoform) and CYP1A2mRNA (typical liver isoform) expression in differentiation lineages – SB and DMSO, namely quick CYP1A1mRNA appearance in first days of differentiation of fetal and adult cells, its fast disappearance during differentiation as well as higher values of CYP1A2mRNA expression in cells of young rats than of old ones – they underline developmental differences in control of transcription existing between both isoforms [38]. It seems clear that they can appear not only in differentiating fetal hepatoblasts but also in oval cells differentiating during liver regeneration. The comparison of dynamics of hepatic CYP1A2mRNA expression in control rats and those treated with CDE shows that this expression to a comparable degree characterizes differentiating cells of both subgroups, but it can achieve higher levels in cells of animals fed on diet.

Also the expression of mRNAs for CYP2B1/2 and CYP3A1 showed age dependence in both differentiation lineages. In contrast to old rats, in the cells of young rats and fetuses, this expression appeared quickly and reached high levels. One could therefore expect that in regenerating liver of old rats, parenchymal cells raising in *de novo* as a result of activation of oval cells will show the lower expression of the CYP2B1/2 and CYP3A subfamily than in young rats. The lack of specific for males CYP3A2mRNA expression in both differentiation lineages, can indicate participation of extrahepatic factors in its formation. Presumably one from the most important is the growth hormone [39].

The opposite situation we observed comparing CYP2E1mRNA expression in DCLs of cells isolated from livers of young and old rats. In differentiating cells of old rats CYP2E1 the expression was stronger or it appeared earlier than in the cells of young rats. This pattern of expression seemed not to be a result of ethionine action, since ethionine did not exist in primary culture and then in differentiation lineages. Age-dependent changes in a rate of CYP2E1mRNA expression maturing, distinguish this isoform from others.

Taking into consideration the fact that that oval cells can divide in livers of rats continuously fed with CDE diet, it can be presumed that initial low levels of CYP expression as well as described changes of CYP expressions can be a part of unspecific protective mechanism, permitting these cells to defeat cytotoxic effects of different carcinogens and proliferate even in

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their presence. Low expression of phase I enzymes favours detoxification in relation to metabolic activation. It can explain why cultured oval cells are resistant to the toxic effects of exposition to procarcinogens and also they proliferate much better during experimental hepatocarcinogenesis than hepatocytes [34]. On the other hand, elevated proliferative activity in regenerating organ can influence an increase in frequency of initiation carcinogenesis independent on metabolic activation of xenobiotics linked with monooxygenase reactions, because of higher probability of gene mutation occurrence and their accumulation.

Increasing in DCLs expression of P450s is the proof of choosing through a cell the direction of a development to hepatocyte [40-43]. CYPs could be good candidates for hepatocyte-precursor cell markers specific for final stages of differentiation to hepatocytes both in normal fetal and adult regenerating liver. CYP2E1 could be maturing oval cell marker in aged regenerating livers, however it should be noted that liver regeneration dependent on oval cells and taking place in old rats can be joined with quicker activation of CYP2E1-linked monooxygenase reactions in maturing hepatocytes as compared with young ones.

Summary

The applied model of experimental hepatocarcinogenesis enabled to initiate regeneration in the liver of adult rats a process dependent on non-differentiated cells. Application of CDE diet elevated the number of cells exposing both stem and oval cell markers in young and old rats. It was also found that the normal rat liver may be a source of non-differentiated cells. *in vitro* differentiation of these cells isolated from CDE-treated rats was earlier as compared to non-treated ones. The differentiation potential of oval cells was weaker in old rats than in young ones. Also the sequence of CYP expressions appearance and levels in differentiating cells of intact rat as well as rats treated with CDE diet differed. This was partly connected with the larger percentage of GSTα(+) and GSTπ(+) cells in cultures isolated from rats treated with CDE (different CYPmRNA levels), but it proves also that oval cells after CDE diet reacted differently to factors used in DCLs (different time of CYP expression appearance) than intact cells. The expression of CYP1A2, CYP2B1/2 and CYP3A1 mRNAs was higher in the cells from young rats as compared to old ones. The differentiating hepatocyte-precursor cells isolated from old rats showed delay of the time of beginning of mRNA synthesis for CYP1A2, CYP2B1/2 and CYP3A1 in relation to young rats. The expression of CYP2E1mRNA was higher in the differentiating cells from old rats and can be elevated in hepatocytes coming *de novo* in aged individuals.

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