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Expression of MRP2 and MDR1 Transporters and Other Hepatic Markers in Rat and Human Liver and in WRL 68 Cell Line

D. ČÍŽKOVÁ[1], J. MOKRÝ[1], S. MIČUDA[2], J. ÖSTERREICHER[3], J. MARTÍNKOVÁ[2]

[1]Department of Histology and Embryology, [2]Department of Pharmacology, Faculty of Medicine in Hradec Králové, Charles University, and [3]Department of Radiobiology, Purkyně Military Medical Academy, Hradec Králové, Czech Republic

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Summary

Here we describe a comparative study of phenotypic properties of hepatic cells *in situ* and *in vitro*. We analyzed the expression levels and distribution patterns of ABC transporters MRP2 and MDR1, pan-cytokeratin, cytokeratin 18, albumin, alpha-fetoprotein and the specific hepatocyte marker OCH1E5 in the fetal and adult rat as well as human liver tissue and in human fetal hepatocytes of WRL 68 cell line using peroxidase immunohistochemistry or immunofluorescence. Transporters MRP2 and MDR1 were expressed in all examined liver tissues, except rat ED13 embryo. The immunopositivity of these proteins was localized to the canalicular membrane of differentiating and mature hepatocytes but in the later developmental stages and in the adult liver tissues it was also found in the apical membrane of cholangiocytes. In WRL 68 cells, MRP2 and MDR1 immunoreactivity appeared after 5-6 days of cultivation and both transporters were fully expressed in the plasmalemma and in the cytoplasm 9 days after the passage. In conclusion, we observed only moderate variances reflecting diverse ontogenetic phases between the fetal and adult liver tissue. To study functions of hepatocytes *in vitro*, WRL 68 cells have to differentiate prior to the examination. Our findings indicate that WRL 68 cells can undergo differentiation *in vitro* and their antigenic profile closely resembles hepatocytes in the human liver.

Key words

MRP2 protein • MDR1 protein • WRL 68 cell line • Liver • Immunohistochemistry

Introduction

Many biological studies investigating hepatocyte functions are performed using *in vivo* models in experimental animals. However, *in vitro* experiments have recently been preferred and widely used. The possibility to examine human liver cells is one of the reasons. For the study of hepatocytes, primary cultures are often used. Nevertheless, they have many disadvantages: heterogeneity, a rapid loss of the hepatocytic phenotype, a limited replicating capacity etc. (reviewed by Strick-Marchand and Weiss 2003).

Therefore, human adult or fetal hepatic cell lines are a good tool for the research of hepatocytic functions (Gutierrez-Ruiz *et al.* 1994, 1995). Here we describe a comparative study of phenotypic properties of hepatocytes *in situ* and *in vitro* with the aims to define the differences between 1) the human and rat liver tissue, 2) the fetal and adult liver tissue and 3) human fetal hepatocytes *in situ* and WRL 68 cells *in vitro*.

Markers feasible for immunophenotypization of hepatocytes were chosen and subsequently, the detection of these markers was performed in human and rat fetal as well as adult liver tissues and in human fetal hepatocytes of the WRL 68 cell line. We analyzed the expression levels and distribution patterns of MRP2 and MDR1 transporters, pan-cytokeratin, cytokeratin 18, plasma proteins albumin and alpha-fetoprotein and the specific hepatocyte marker OCH1E5. Concomitantly, we observed the expression of the above-mentioned markers at different time intervals.

Material and Methods

Histology

To acquire the rat fetal liver tissue, female Wistar rats were mated with males overnight and the following day was designated as embryonic day 0 (ED0). ED13 and ED19 rat embryos were obtained after a medial laparotomy in deeply anesthetized (1:1 mixture of 2 % xylazine and 5 % ketamine; 0.0015 ml/g, i.p.) pregnant rats. Thereafter, pregnant rats were killed by anesthetic overdose. The livers of adult rats were obtained using a similar procedure, i.e. after a medial laparotomy carried out in deeply anesthetized animals. Specimens of the human adult and fetal liver tissue were taken from the archive of formalin-fixed paraffin-embedded blocks in the Department of Histology and Embryology.

Rat fetuses as well as adult rat livers were immersed into a neutral buffered formalin solution for 72 h at room temperature. Tissue blocks were then dehydrated in increasing alcohol concentrations and embedded in paraffin. Seven-micron-thick coronal serial sections were cut and attached to the slide with alum gelatine. Every tenth slide was stained with hematoxylin and eosin and the other were processed for immunohistochemistry.

Cell culture

We obtained the WRL 68 cell line from ATCC (USA) after the 82nd passage. Cells were routinely cultivated in EMEM medium (BioWhittaker, Cambrex, USA) supplemented with 10 % fetal bovine serum (Gibco, Invitogen USA) and antibiotics (penicillin 100 U/ml, streptomycin 100 µg/ml; Sigma, Czech Republic) at 37 °C in a humid atmosphere of 95 % air and 5 % CO_2. To maintain the immature phenotype of fetal hepatocytes, cells were grown in culture flasks (Nunc, Denmark) in the form of a monolayer and passaged twice a week in the split ratio 1:15. Special PetriPERM dishes (Vivascience, Germany) with only 25 µm thick gas-permeable plastic film instead of the usual plastic bottom were used to permit cell differentiation. Three-dimensional structures formed in these dishes and all cells including elements in the lowest layers retained their viability due to the possibility of oxygen and carbon dioxide exchange.

The test of viability of the WRL 68 cell line under our conditions was performed using propidium iodide staining. On day 0, cells were thawed and 4 h following their seeding, the medium was washed with PBS and propidium iodide (50 µg/ml) was added for 10 min. The dye was then washed with PBS and the cell culture was examined in Nikon TE 300 Eclipse microscope. Propidium iodide positive nuclei were counted with the use of fluorescent microscopy and the number of all cells was measured in the same microscopic field under the phase contrast. This procedure was repeated to evaluate cell viability 24 h, 48 h, 76 h and 96 h after thawing of the cells.

Immunohistochemistry and immunocytochemistry

For immunohistochemistry, paraffin-embedded sections of the human and rat liver were deparaffinized with xylene and rehydrated in decreasing ethanols to water. To reestablish the original conformation of epitopes modified after fixation, we performed antigen retrieval using a microwave (in sodium citrate solution for 3 x 5 min at 700 W) or enzyme digestion (0.2 % pepsin solution, 30 min in 37 °C). Endogenous peroxidase was then blocked in 1 % H_2O_2 (3 x 10 min) followed by incubation in 5 % normal donkey serum (Jackson ImmunoResearch Laboratories, USA). The following primary antibodies were used: mouse anti-hepatocyte, clone OCH1E5 (DakoCytomation, Denmark), rabbit anti-alpha-1-fetoprotein (Dako, Denmark), mouse anti-PANcytokeratin, clone KL1 (Immunotech, France), mouse anti-cytokeratin 18, clone DC 10 (Dako, Denmark), mouse anti-P-glycoprotein, clone C219 (Signet, USA) and mouse anti-cMOAT/MRP2, clone M_2III-6 (Signet, USA). Sections were incubated with

these primary antibodies overnight at 4 °C and after washing in PBS, they were exposed to anti-mouse or anti-rabbit secondary biotinylated antibodies (Jackson ImmunoResearch Laboratories, USA) for 45 min at room temperature. After rinsing, sections were incubated with streptavidin labelled with horseradish peroxidase (Sigma, Czech Republic) for 45 min and then the reaction was developed using DAB (3,3-diaminobenzidine tetrahydrochloride, Fluka, Czech Republic). Sections processed with omission of the primary antibody were used as controls. Sections were dehydrated, mounted in DPX and examined by the light microscope.

In several sections, catalyzed signal amplification was used to enhance the immunohistochemical signal. We performed one amplification cycle with the use of biotinylated tyramine.

For immunofluorescent staining, WRL 68 cells harvested from cultures were fixed with a 1:1 mixture of ice cold methanol and acetone for 15 min. After washing in PBS, the above-mentioned primary antibodies were used (overnight, 4 °C), rinsed with BPS and followed by the incubation with anti-mouse or anti-rabbit secondary antibody conjugated with fluorochrome Cy3 (Jackson ImmunoResearch Laboratories, USA). Specimens were then mounted in polyvinyl alcohol/glycerine with 1,4-diazabicyclo[2.2.2]octane (DABCO, Molecular Probes) and examined under a fluorescent microscope.

Results

Fetal rat liver

In the liver of ED13 rat embryos we detected a weak immunopositivity for Mrp2 (multidrug resistance associated protein 2, cMOAT, canalicular multiorganic anion transporter) in canalicular membranes of developing hepatocytes. Mrp2-positive hepatocytic membranes formed walls of bile canaliculi. The hepatic cytoplasm did not show any signal of this transporter. In differentiating hepatocytes of ED19 rat embryos we noted a strong immunoreactivity for Mrp2 localized to canalicular membranes, whereas a weak signal was distinguished in their cytoplasm. Other components of the liver tissue of both developmental stages did not express this glycoprotein.

We did not observe any immunoreactivity for Mdr1 (multidrug resistance protein 1, P-glycoprotein) in the liver of ED13 rat embryos. However, in ED19 embryos this transporter was already moderately expressed in canalicular membranes and weakly in the cytoplasm of maturing hepatocytes. We did not detect the Mdr1 protein in other cells of the liver tissues.

Adult rat liver

In the liver of adult rats, the immunoreactivity for Mrp2 (cMOAT) was localized in the bile canaliculi membrane. The cytoplasm, the nucleus and the remaining plasmalemma of hepatocytes were devoid of the staining, as well as the connective tissue and vessels of the liver parenchyma. The intensity of the immunostaining for Mrp2 in canalicular membrane was generally very high. When we compared areas in the vicinity of portal spaces and areas surrounding the central vein, there was no difference in the intensity and distribution of the Mrp2 signal. We also detected this transporter in the apical membranes of cholangiocytes forming interlobular bile ducts.

Mdr1 (P-glycoprotein) was also expressed only in the bile canaliculi membrane of hepatocytes; all the other subcellular components were immunonegative. The remaining structures of the liver tissue did not show any positivity for Mdr1, which confirmed a high specificity of the immunostaining. The signal for this transporter was significantly weaker than that for Mrp2, but its spatial distribution was very similar.

Fetal human liver

In the liver of 14-week-old human fetuses, MRP2 was detected in the walls of bile canaliculi, whereas the cytoplasm of differentiating hepatocytes remained immunonegative. At a later stage of intrauterine development (19 weeks) this transporter was strongly expressed in canalicular membranes of immature hepatocytes, while a weak signal appeared in the cytoplasm of these cells (Fig. 1A). It is noteworthy that we observed a strong immunopositivity in the apical membrane of developing cholangiocytes (Fig. 1A). All other structures of the developing liver parenchyma showed no immunoreactivity for this protein.

We distinguished the signal for MDR1 in the liver tissue of 14-week-old human fetuses in canalicular membranes of developing hepatocytes, but the level of its intensity was extremely low. In the hepatic tissue of 19-week-old fetuses MDR1 was moderately expressed in the wall of bile canaliculi and a weak signal of this transporter was also revealed in the cytoplasm of maturing hepatocytes (Fig. 1B). MDR1 was also detected in the apical cytoplasm of differentiating cholangiocytes (Fig. 1B).

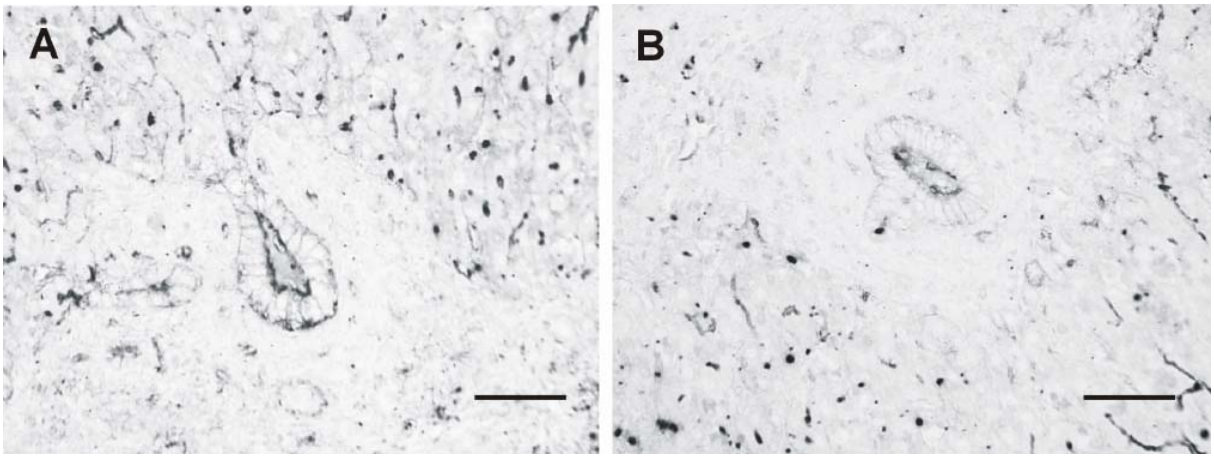

Fig. 1. MRP2 and MDR1 expression in the liver of a 19-week-old human fetus. MRP2 (**A**) is expressed in canalicular membrane of developing hepatocytes and also in the apical membrane of maturing cholangiocytes of an interlobular bile duct. MDR1 (**B**) reveals an almost identical staining pattern as MRP2. MDR1 is detected in canalicular membrane of fetal hepatocytes and in apical membrane of fetal cholangiocytes. Scale bars A, B, 50 μm.

The human hepatocyte marker OCH1E5 was expressed in differentiating hepatocytes in the liver of 14-week-old as well as of 19-week-old human fetuses with a high intensity.

The immunoreactivity for pan-cytokeratin in the liver tissue of 14-week-old and 19-week-old human fetuses showed a week signal throughout the cytoplasm of immature hepatocytes, whereas the developing cholangiocytes situated around the portal spaces revealed a strong immunopositivity in their cytoplasm.

The intermediate filament cytokeratin 18 was expressed in the cytoplasm of maturing hepatocytes and cholangiocytes in the liver of 14-week-old human fetuses. The immunostaining was almost homogeneous, there were only small differences between hepatocytes and cholangiocytes. In the liver of 19-week-old human fetuses the highest levels of cytokeratin 18 were detected in the cytoplasm of cholangiocytes, especially in those surrounding portal spaces. This protein was also observed in the cytoplasm of maturing hepatocytes, where the signal was weaker than in cholangiocytes, but borders among hepatocytes were visible very clearly thanks to strong immunopositivity. If we compared the intensity, the signal of cytokeratin 18 was stronger than that of pan-cytokeratin in the fetal human liver tissue.

The endodermal marker alpha-fetoprotein was expressed only in the cytoplasm of differentiating hepatocytes in the liver of both studied developmental stages of the human fetus.

We observed a weak immunoreactivity for albumin only in the cytoplasm of maturing hepatocytes in human liver tissue of 14-week-old and 19-week-old fetuses.

In sections with immunostaining for OCH1E5, pan-cytokeratin, cytokeratin 18, alpha-fetoprotein and albumin there was no immunopositivity in nuclei of hepatocytes and cholangiocytes, in the connective tissue and vessels.

Adult human liver

MRP2 (cMOAT) was detected in the bile canaliculi membrane of hepatocytes and in the apical membrane of cholangiocytes (Figs 2A, 2C, 2E). A weak immunoreactivity of this transporter was revealed in the cytoplasm of hepatocytes, while no signal was observed in any other structures of the liver tissue. The distribution of MRP2 in the examined liver tissue was almost homogeneous. There was no significant difference between the expression of this protein in the walls of bile canaliculi in the vicinity of portal spaces and around the central vein area.

MDR1 was strongly restricted to the canalicular membrane and weakly to the cytoplasm of hepatocytes (Figs 2B, 2D). The immunoreactivity for this protein was also situated to the apical membrane of cholangiocytes (Figs 2D, 2F). The signal was not present in any other structures of the liver tissue. The intensity of immunostaining of this protein was lower than that of MRP2, whereas the spatial distribution of both these ABC transporters was identical.

The immunopositivity for the marker OCH1E5 displayed a granular staining pattern, which was present throughout the cytoplasm of hepatocytes. There was a small difference in the intensity of immunostaining

between hepatocytes.

Pan-cytokeratin was weakly expressed in the cytoplasm of hepatocytes, but several immunonegative hepatocytes were also present. However, strong immunoreactivity was detected in the cytoplasm of cholangiocytes, which allowed cuboidal epithelial cells Herring canals to be distinguish as well as interlobular bile ducts lined with columnar epithelial cells localized in portal spaces.

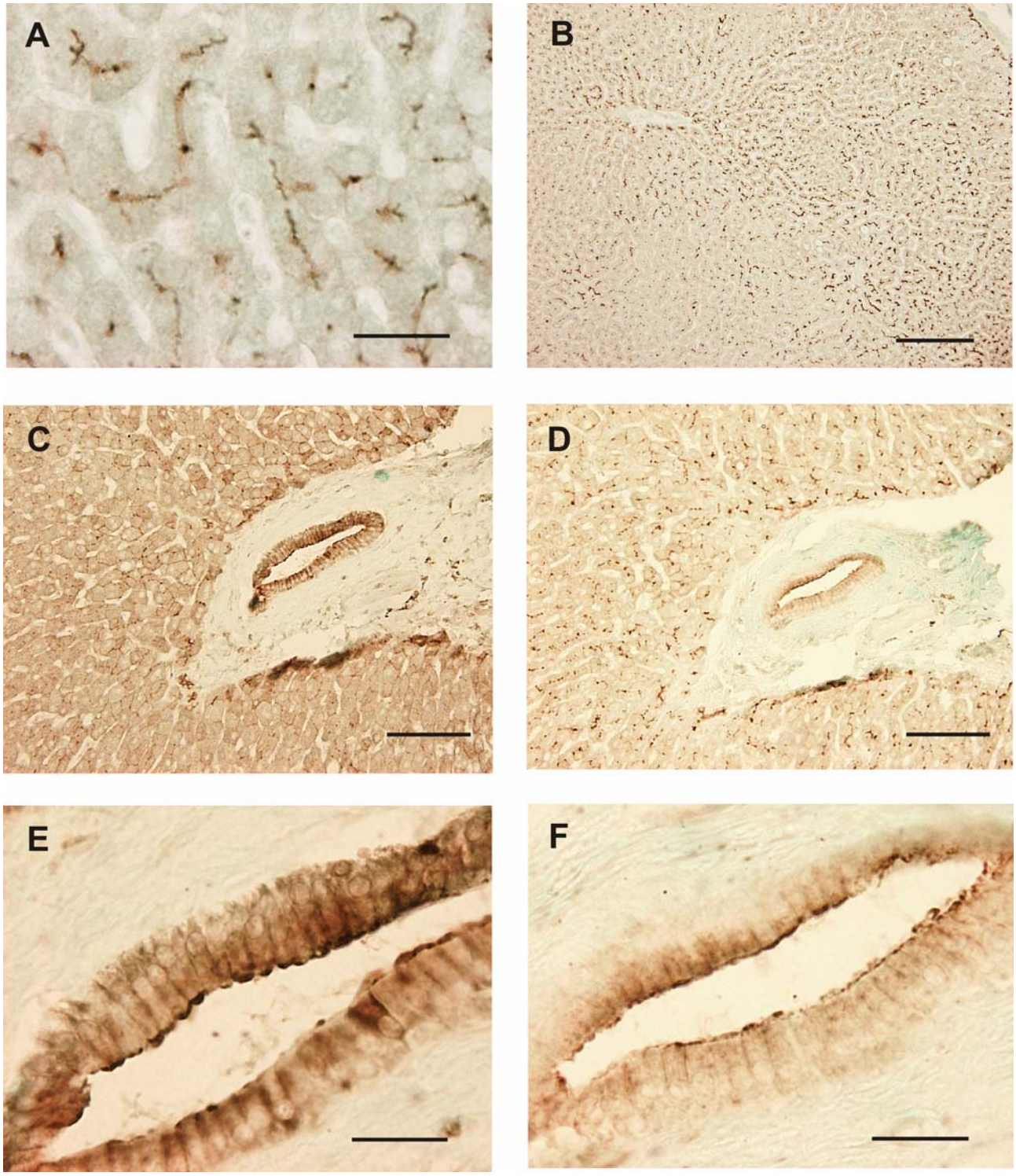

Fig. 2. MRP2 and MDR1 expression in the human adult liver tissue. MRP2 is expressed in the canalicular membrane of hepatocytes so intensively, that its immunopositivity visualizes bile canaliculi pathways (**A, C**). MRP2 is also detected in the apical membrane of cholangiocytes forming interlobular bile ducts (**C, E**). The immunoreactivity for MDR1 is visible in the canalicular membrane of hepatocytes (B, D) and in the apical membrane of cholangiocytes (**D, F**) similarly as MRP2, only the intensity of MDR1 is lower than that of MRP2. Scale bars A, E, F, 25 µm; B, 200 µm; C, D, 100 µm.

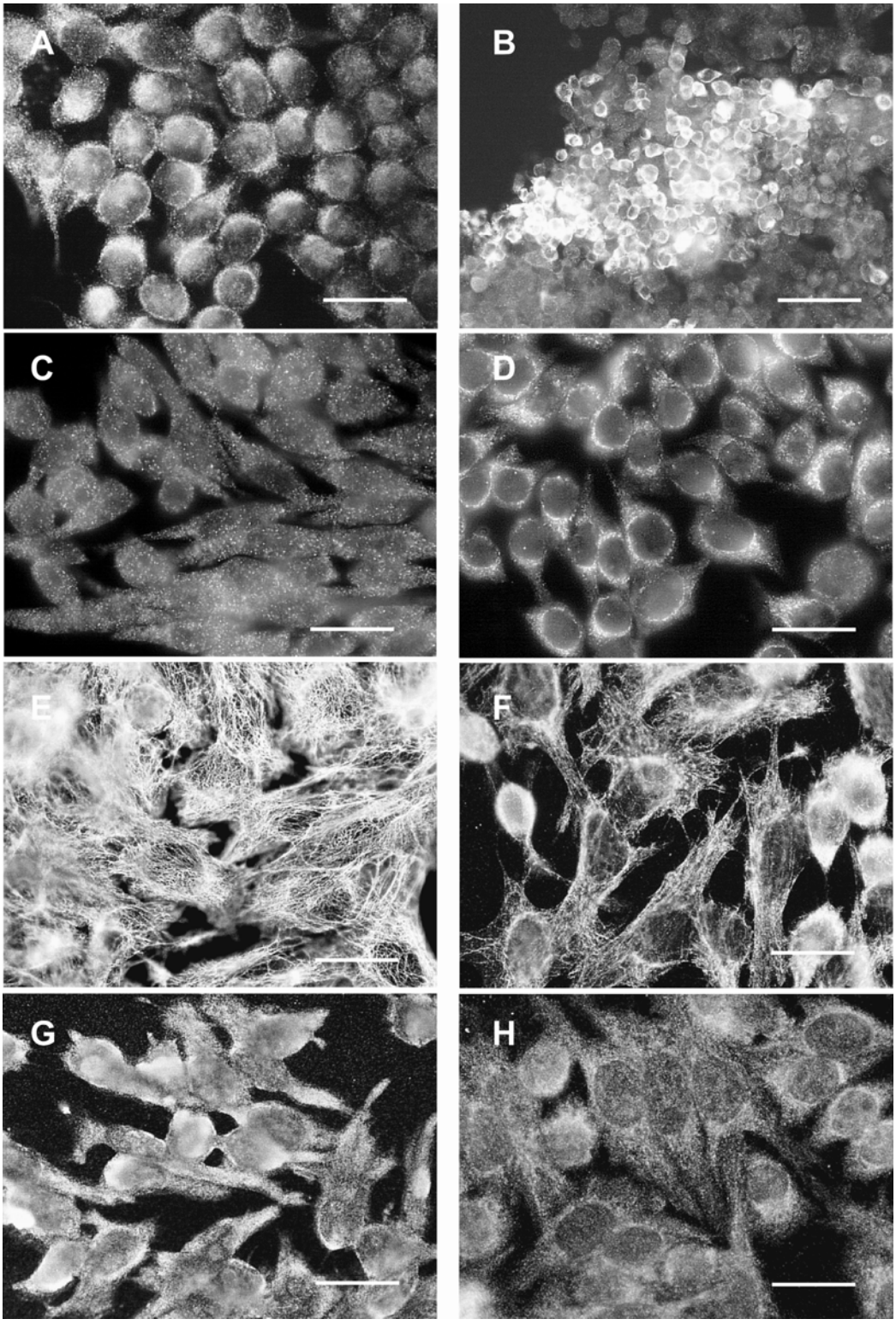

Fig. 3. Expression of MRP2, MDR1, OCH1E5, pan-cytokeratin, cytokeratin 18, alpha-fetoprotein and albumin in human fetal hepatocytes of WRL 68 cell line 5-6 days *in vitro* (DIV). MRP2 is expressed in the plasmalemma and the cytoplasm of WRL 68 cells. The immunopositivity reveals a punctuate staining pattern giving evidence that these elements are still not polarized (5 DIV; **A**). In the areas where cells overgrow each other MRP2 is strongly expressed, whereas in areas of the monolayer the signal is less intensive. (6 DIV; **B**). The punctuate immunoreactivity for MDR1 in WRL 68 cells is weak and situated to the plasmalemma and the cytoplasm (5 DIV; **C**). The signal of human hepatocyte marker OCH1E5 is localized to the cytoplasm of WRL 68 cells and appears a granular staining pattern. (5 DIV; **D**). The immunostaining for pan-cytokeratin shows the filamentous structure of the mixture of the cytokeratin intermediate filaments in the cytoplasm of WRL 68 cells (5 DIV; **E**). The staining pattern of intermediate filament cytokeratin 18 is very similar to that of pan-cytokeratin, only the network of filamentous proteins is poorer (5 DIV; **F**). The punctuate immunopositivity for alpha-fetoprotein is dispersed throughout the cytoplasm of WRL 68 cells (5 DIV; **G**). The immunoreactivity for albumin reveals as a large quantity of very small dots localized to the cytoplasm of WRL 68 cells (5 DIV; **H**). Scale bars, 25 μm.

We observed an expression of the intermediate filament cytokeratin 18 in hepatocytes and also in cholangiocytes. In hepatocytes, the signal of this protein was detected in the cytoplasm, but the immunopositivity was accentuated under the membrane contacting neighboring hepatocytes. The strongly immunopositive cytoplasm of cholangiocytes visualized the structure of intrahepatic bile ducts lined with cuboidal or columnar epithelial cells.

Alpha-fetoprotein was expressed only in the cytoplasm of hepatocytes in an extremely low intensity. No positive signal was visible in cholangiocytes.

The immunoreactivity for albumin was localized in the cytoplasm of hepatocytes, while cholangiocytes remained immunonegative.

We did not observe any signals in the nuclei of hepatocytes and cholangiocytes, in the connective tissue and blood vessels in sections processed for anti-OCH1E5, pan-cytokeratin, cytokeratin 18, alpha-fetoprotein and albumin immunostaining.

WRL 68

Human fetal hepatocytes of the WRL 68 cell line revealed a normal phenotype, they were passaged in a 1:15 split ratio and frequent mitotic divisions were present. These facts reflected the good condition of this culture. Cells proliferated briskly; 4 h after thawing, there were 2 624 cells/100 mm^2 and their quantity increased exponentially in the course of the cultivation. 96 hours after thawing, the number of cultivated cells increased to the value 102 426 cells/100 mm^2. Without passaging, they reached 100 % confluence and started to grow over each other. Elements localized directly on the bottom of the flask covered by other cells started to die because of a deficit in oxygen and nutrients. After the 5^{th} day after passaging we found an increasing number of dead cells floating in the medium. To avoid this undesirable phenomenon and to maintain our culture for a longer period of time allowing cell differentiation, we began to cultivate WRL 68 cells in special PetriPERM dishes, which had only a 25 μm thick gas-permeable plastic film instead of the usual plastic bottom. In these dishes, stratified three-dimensional structures were formed and elements localized in lower layers retained their viability due to the possibility of oxygen and carbon dioxide exchange.

We performed the test of viability using propidium iodide staining of dead cells. The percentage of dead propidium iodide-positive cells in the culture was approximately 1 % and remained constant. Therefore, the viability of human fetal hepatocytes of the WRL 68 cell line reached about 99 % in our conditions.

On day 3 after the passage, WRL 68 cells did not show any signal for MRP2. The immunoreactivity for this marker appeared on days 5-6 *in vitro* (Fig. 3A), while on day 9 all hepatocytes expressed this protein. Individual hepatocytes of the same age *in vitro* differed in the intensity of the signal. Some cells displayed intensive immunopositivity, whereas the other showed only a weak signal. Hepatocytes with a high expression of MRP2 were localized to the areas, where cells grew over each other. Correspondingly, cells forming a monolayer showed a weak signal (Fig. 3B). The immunoreactivity for this transporter was situated to the plasmalemma, a weak signal could also be detected in the cytoplasm of cultivated hepatocytes.

The expression pattern of the MDR1 was similar to that of MRP2. On day 5-6 after the passage, a weak signal was observed (Fig. 3C) and an intense immunoreactivity was detected on day 9. Differences in the signal intensity among individual hepatocytes were not so pronounced as that noted in MRP2 expression. Nevertheless, areas with higher MDR1 expression were observed at sites, where cells grew over each other, while areas where cells formed a monolayer exhibited a low expression. MDR1 was localized to the plasmalemma and the cytoplasm of hepatocytes, where it showed a punctuated staining pattern.

A specific hepatocyte marker OCH1E5 was detected in the cytoplasm of *in vitro* hepatocytes (Fig. 3D). The immunopositivity revealed its granular staining pattern. There was no difference in the expression level among cultivated elements.

The immunostaining of intermediate filaments pan-cytokeratin (Fig. 3E) and cytokeratin 18 (Fig. 3F) revealed the filamentous structure of the cell cytoskeleton. The network of pan-cytokeratin was richer than that of cytokeratin 18. Except for this difference, the staining pattern of these two intermediate filaments was almost identical.

Hepatocytes expressed albumin (Fig. 3G) as well as alpha-fetoprotein (Fig. 3H). Both proteins were found in the cytoplasm of cells.

Discussion

MRP2 (rat Mrp2) and MDR1 (rat Mdr1 or P-glycoprotein) represent the group of ABC transporters (ATP-binding cassette proteins) which are transmembrane proteins permitting transport or excretion of endogenous and exogenous compounds out of the cell. These transporters are responsible for multidrug

resistance of several drugs, such as cytostatics and antibiotics (reviewed by Hooiveld *et al.* 2001).

MRP2 (Mrp2) is a product of the human *MRP2* or rodent *Mrp2* gene. In normal tissues, MRP2 is highly expressed in the canalicular membrane of hepatocytes, in the apical membrane of enterocytes and proximal tubule cells. This protein functions as a multispecific organic anion transporter which transports e.g. glutathione S-conjugates such as bilirubin- and estrogen-glucuronides. MRP2 is not expressed in patients with Dubin-Johnson syndrome (an inheritable disorder that is associated with deficient biliary secretion of anionic conjugates, including bilirubin glucuronides) (König *et al.* 2003). We observed the immunoreactivity of this glycoprotein in the rat fetal liver tissue in ED13 and ED19 embryo and in the liver of 14-week-old as well as 19-week-old human fetus. The signal was localized to canalicular membranes of differentiating hepatocytes and in the later developmental stage, a weak immunopositivity was noticed also in the cytoplasm of these elements. Moreover, in 19-week-old human fetus MRP2 was expressed in the apical membrane of developing cholangiocytes forming bile ducts in the connective tissue of portal spaces. Mrp2 expression was identified during rat development in ED16 (Zinchuk *et al.* 2002), but earlier embryonic stages were probably not examined. Therefore, according to our findings, Mrp2 is already expressed in the rat ED13 embryo. In developing rat embryo, rough endoplasmic reticulum and Golgi apparatus are fully formed in differentiating hepatocytes in ED12 and at this stage the synthesis of secreted proteins already proceeds (Luzzatto 1981, Medlock and Haar 1983, reviewed by Duncan 2003). Moreover, bile canaliculi that represent the canalicular surface of hepatocyte can be detected around day 12 of gestation in the rat (Luzzatto 1981, Wood 1965, reviewed by Duncan 2003). Therefore, morphological and physiological groundings for the Mrp2 synthesis in ED13 rats are already present. MRP2 immunoreactivity in human fetuses was not studied thoroughly. Expression of this transporter in canalicular membrane in the liver of 14-week-old fetuses and in the canalicular membrane and also in apical membrane of cholangiocytes in the hepatic tissue of 19-week-old fetuses could represent new findings extending our knowledge on properties of MRP2 protein. The intensity of the MRP2 signal was generally higher in the older fetal liver tissues. Thus, the expression of MRP2 increased in the course of hepatocyte differentiation, which is in concert with the results of Zinchuk *et al.* (2002). MRP2 was expressed in adult liver tissues in rats as well as in man in an identical expression pattern. The immunopositivity obviously showed the structure of bile canalicular system due to the immunostaining of walls of the bile canaliculi. We observed a strong MRP2 signal also in apical membrane of cholangiocytes in interlobular bile ducts. Sandusky *et al.* (2002) who studied expression of MRP2 in normal human tissues did not find any immunopositivity in bile ductules. On the other hand, Rost *et al.* (2001) described that MRP2 is expressed in human gallbladder epithelia. Several researchers dealt with MRP2 expression in hepatocytes, but they did not pay their attention to cholangiocytes.

MDR1 (Mdr1a/1b) is encoded by the human *MDR1* or rodent *Mdr1a/1b* gene. Under physiological conditions, MDR1 is expressed at low levels in canalicular membranes of hepatocytes, in brush border membranes of enterocytes and proximal tubule cells, in the blood-brain barrier, etc. A major function of this transporter is the protection of hepatocytes against toxic effects of many substances. Furthermore, it has additional functions, e.g. transport of endogenous agents, such as steroid hormones or secretion of cytokines (reviewed by Hooiveld *et al.* 2001). We did not detect Mdr1 in ED13 rat liver, but in ED19 rat this protein was already expressed in the canalicular membrane of hepatocytes. Thus, Mdr1 in ED13 rat liver tissue was not expressed or it was present in the undetectable amount. In the human tissue, MDR1 was detected in 14-week-old fetus in canalicular membrane of differentiating hepatocytes and in 19-week-old animals in the same structures and also in apical membrane of cholangiocytes in developing portal spaces. MDR1 gene expression was found in the liver in the 11^{th} week of gestation (Lecureur *et al.* 2000) and van Kalken *et al.* (1992) identified this transporter in walls of bile canaliculi in 14^{th} week of intrauterine development. In one specimen they also observed MDR1 immunopositivity in bile ductules but not in larger bile ducts. In the adult rat and human livers, MDR1 was detected in the same localizations. The immunoreactivity was observed in the canalicular membrane of hepatocytes and in apical membrane of cholangiocytes forming interlobular ducts. Thiebaut *et al.* (1987) studied the localization of this transporter in normal human tissues, they detected MDR1 on the biliary surface of hepatocytes and on the apical surface of small biliary ductules. In general, the intensity of MDR1 signal was lower than that of MRP2, which corresponds to recent findings described in the literature.

In human fetal hepatocytes of the WRL 68 cell line, MRP2 and MDR1 were noticed after 5-6 days *in vitro* and the strongest signal was detected in areas, where

cells overgrew each other. Therefore, immature hepatocytes (the 3rd day *in vitro*) did not express these transporters or they expressed them at very low levels. During the cell differentiation, when hepatocytes acquired new intercellular contacts, they started to synthetize MRP2 and MDR1 transporters with increasing intensity. The immunopositivity of both proteins was localized to the membrane and a weak signal was also observed in the cytoplasm of examined elements. This indicated that fetal hepatocytes of the WRL 68 cell line cultivated *in vitro* were still not polarized. A weak immunoreactivity for both examined transporters in developing as well as mature hepatocytes *in situ* and *in vitro* in their cytoplasm could be interpreted by the presence of these proteins and their precursors in the endoplasmic reticulum and Golgi apparatus during their synthesis.

The human hepatocyte marker OCH1E5 allows to distinguish hepatocytes from other cell types. This marker is not expressed in cholangiocytes or in non-parenchymal liver elements (Wennerberg *et al.* 1993). We could detect this marker only in human tissues, because the used antibody did not cross-react with the rat tissue. In the fetal as well as in the adult human liver tissue, we observed OCH1E5 immunopositivity in the identical expression pattern. The signal was granular and localized to the cytoplasm of developing or mature hepatocytes. In fetal human hepatocytes of the WRL 68 cell line we detected the same granular immunoreactivity throughout their cytoplasm.

Pan-cytokeratin represents a mixture of 13 cytokeratins. Its signal is detected in hepatocytes as well as in cholangiocytes. The intermediate filament cytokeratin 18 is also present in both the above-mentioned liver cell types (van Eyken *et al.* 1987). We observed the expression of these two markers in all examined liver tissues as well as in cultivated fetal human hepatocytes of the WRL 68 cell line. In general, the signal of both markers is strongest in the cytoplasm of cholangiocytes, a weaker one was noticed in the cytoplasm of hepatocytes. Interestingly, an accentuated cytokeratin 18 immunopositivity was observed under plasma membranes contacting neighboring hepatocytes. This localization corresponds to the anchorage of intermediate filaments to the plasmalemma in the place of *zonula occludens* intercellular junctions. In human fetal hepatocytes grown *in vitro*, the immunostaining revealed the obvious filamentous structure of intermediate filament proteins.

Alpha-fetoprotein (AFP) is the major serum protein during development. In humans, this protein is detectable in the liver at 26 days after ovulation (at the end of the 4th week of intrauterine development). After birth, the synthesis of AFP decreases dramatically and only trace amounts are expressed in the adult liver (Jones *et al.* 2001). We detected this protein in the cytoplasm of human fetal hepatocytes *in situ* as well as of WRL 68 cells *in vitro*. In adult livers, an extremely weak immunoreactivity for AFP was localized to the cytoplasm of hepatocytes.

Albumin, a plasma protein synthesized in hepatocytes, is a useful marker for hepatocyte characterization. It is not expressed in cholangiocytes or in any other liver elements. In the rat embryo, hepatocytes produce albumin since ED12 (Luzzatto 1981). We observed weak albumin expression in the cytoplasm of developing hepatocytes in the liver tissue of ED13 rat and 14-week-old human fetus. In the course of liver differentiation, the intensity of immunostaining increased and the highest levels were detected in adult hepatocytes.

In conclusion, we did not notice any significant difference in the expression pattern of chosen markers between the rat and human liver tissues. If we compared fetal and adult liver tissues, there were moderate variances reflecting diverse ontogenetic phases. However, the most noteworthy was the comparison of human fetal hepatocytes *in situ* and WRL 68 cells *in vitro*. Morphological properties and the antigenic profile of human fetal hepatocytes *in situ* and *in vitro* are according to our findings closely similar. Our additional findings, which have not been described in the literature, include MRP2 expression in the human fetal liver tissue, MRP2 immunopositivity in the human adult liver tissue in the apical membrane of cholangiocytes forming interlobular bile ducts and, last but not least, this study provides the first description of MRP2 and MDR1 expression in the human fetal hepatic cell line WRL 68. For the study of hepatocyte functions *in vitro*, cells have to differentiate and WRL 68 hepatocytes have the ability to undergo differentiation similar to the hepatocytes in the developing liver tissue.

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Reprint requests

D. Čížková, Department of Histology and Embryology, Faculty of Medicine, Charles University, Šimkova 870, 500 38 Hradec Králové. Czech Republic. E-mail: cizkovad@lfhk.cuni.cz