Expression and regulation of ABC transporter genes during liver regeneration
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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2002

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Chapter 3

ATP-binding cassette transporter gene expression in rat liver progenitor cells

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SUBMITTED
3.1 Abstract

Liver damage results in activation of progenitor cells when proliferation of hepatocytes is suppressed. Progenitor cells are characterized by the expression of specific proteins like Thy-1. We studied the expression of ATP-binding cassette (ABC) transporter genes in rat liver progenitor cells. The progenitor cell compartment was induced by treating rats with 2-acetylaminofluorene (2-AAF) followed by partial hepatectomy (PHx). mRNA levels of ABC transporter genes were determined by real time detection RT-PCR. 2-AAF/PHx-treated animals showed increased hepatic mRNA levels of the genes encoding multidrug resistance protein \textit{Mdr1b} and multidrug resistance protein associated proteins \textit{Mrp1} and \textit{Mrp3}. Immunohistochemistry demonstrated expression of Mrp1 and Mrp3 in progenitor cells and of Mdr1b in periportal hepatocytes. To quantify ABC transporter expression in separate cell types, isolated liver cell fractions were analyzed. Freshly isolated Thy-1 positive cells and cultured RLE \ensuremath{\varphi}13 cells, both prototypes for hepatic progenitor cells, highly expressed \textit{Mrp1} and \textit{Mrp3} mRNA, while the hepatocyte-specific transporters \textit{Mdr2}, \textit{Bsep}, \textit{Mrp2}, and \textit{Mrp6} were minimally expressed. The expression pattern of ABC transporters resembled that of cholangiocytes except for \textit{Abca1}, which was expressed in Thy-1 positive cells and RLE \ensuremath{\varphi}13 cells, but not in cholangiocytes. In conclusion, as for ABC transporters, hepatic progenitor cells bear more resemblance to cholangiocytes than to hepatocytes. The high expression of Mrp1 and Mrp3 may serve a cytoprotective role in these cells during liver regeneration in conditions of severe hepatotoxicity.
3.2 Introduction

Liver regeneration occurs after loss of liver tissue due to toxic injury or partial hepatectomy (PHx) and involves replication of hepatocytes. Under conditions in which hepatocytes cannot proliferate, liver damage results in the activation of the oval cell compartment. In rats, oval cell proliferation can be achieved by treatment with 2-acetylaminofluorene (2-AAF) in combination with PHx or CCl4 administration. Treatment with 2-AAF followed by PHx results in a decreased expression of Cyclin E and an increased expression of p53 and p21 in hepatocytes. This causes a block in hepatocyte proliferation at the G1/S restriction point. Oval cells are derived from progenitor cells located in the canals of Hering. These cells escape from the 2-AAF-induced blockade and proliferate in the 2-AAF/PHx model. After hepatic injury, their proliferation reaches a maximal level at 9-11 days post hepatic injury. Oval cells resemble fetal hepatic cells as they express fetal markers such as α-fetoprotein and Thy-1 at high levels and are considered to be precursors of hepatocytes as well as cholangiocytes.

Oval cells are only activated after severe liver damage and are supposed to represent a back-up mechanism for hepatic regeneration. We hypothesize that, in view of their critical role in hepatic repair after excessive damage, oval cells should be able to protect themselves against toxic metabolites and xenobiotics. One mechanism of cellular protection could be the expression of efflux pumps that belong to the ATP-binding cassette (ABC) superfamily of membrane transporters.

The ABC transporter family consists of approximately 50 members that have been divided in 7 subclasses (A-G), based on their amino acid sequence homologies. Members of the Abcb (multidrug resistance proteins) subfamily that are expressed in the liver include Mdr1a/b (gene symbol Abcb1a/b, MDR1 in humans), efflux pumps for hydrophobic compounds and chemotherapeutic agents, Mdr2 (Abcb4, MDR3 in humans), a flippase that translocates phosphatidylcholine across the membrane, and Bsep (Abcb11), the export pump for bile salts. These transporters are all located in the canalicular membrane of hepatocytes (reviewed in Hooiveld et al.).

The Abcc (multidrug resistance-associated protein) subfamily consists of 12 members, of which at least four, Mrp1, Mrp2, Mrp3, and Mrp6, are expressed in normal liver (reviewed by Borst and Oude Elferink). Mrp2 (Abcc2) is present in the apical membrane of hepatocytes. Mrp1 (Abcc1) is located in the basolateral membrane but is expressed only at low levels in normal liver. Mrp1 expression is induced in proliferating hepatocytes. Mrp1 and Mrp2 have a similar substrate specificity and transport glutathione S-conjugates, cysteinyl leukotrienes, bilirubin glucuronides, estrogen glucuronides and glutathione disulfide (GSSG). Hepatic expression of Mrp3, Mrp4, and Mrp6 has been reported recently. In normal liver, Mrp3 (Abcc3) is expressed in the basolateral membrane of pericentrally-localized hepatocytes and in cholangiocytes. Mrp3 is able to transport mono- and bivalent bile salts as well as glucuronide conjugates. Its expression is increased during cholestasis and in conditions of conjugated or unconjugated hyperbilirubinemia (Gunn rats and Mrp2-deficient mutant rats). Mrp4 (Abcc4) is an export pump for organic anions, but is also capable of transporting cyclic nucleotides and nucleotide analogues.
In normal liver, Mrp4 is expressed at low level. However, enhanced hepatocellular concentrations of bile salts increase Mrp4 expression in liver. Mrp6 (Abcc6) is expressed at high levels in normal liver, predominantly in the basolateral membrane of hepatocytes. Its transport specificity is less well defined.

A number of these transporters have been linked to cellular protection. Mdr1b functions as efflux pump for toxins, but may also have an anti-apoptotic role. Mrp1 is a transporter of leukotriene C4 and the glutathione conjugate of prostaglandin A2, factors involved in inflammation and cell cycle arrest. Moreover, Mrp1-mediated transport of glutathione disulfide and glutathione conjugated 4-hydroxynonenal suggests that Mrp1 functions as part of the cellular defense system against oxidative stress (reviewed by Renes et al.). Mrp3 may have an important role in protecting cells from endogenous bile salts by extruding these into the blood.

Two other members of the ABC family that may be of special interest in relation to oval cells are Abca1 and Bcrp (Abcg2). Abca1 is highly expressed in fetal liver, where its expression correlates with apoptotic areas. Moreover, Abca1 is ubiquitously expressed in various tissues and organs. It is essential for HDL formation. Its specific function in adult liver is currently unknown. Bcrp is expressed at low levels in the apical membrane of hepatocytes. Expression of Bcrp can, like Mdr1a/b and Mrp1, cause multidrug resistance. Bcrp may have a role in maintaining a dedifferentiated phenotype in a subgroup of hematopoietic stem cells.

We and others have studied the expression of a number of these transporters in regenerating rat liver after 70% partial hepatectomy (PHx). In this model mature hepatocytes start to proliferate to make up for the loss of tissue. Twenty-four hours after PHx, at the peak of DNA replication, hepatocytes show a striking increase in Mdr1b mRNA expression. Mdr2 and Mrp1 are slightly increased after PHx.

The expression profile of ABC transporters in the oval cell compartment is not known. Since that oval cells represent a potential proliferative reservoir of a severely damaged liver, we speculate that these cells must be well protected. We therefore studied ABC transporter expression in hepatic oval cells.

3.3 Materials and methods

Animals

Specified pathogen-free male Wistar and Fisher 344 rats (130-170 g) were purchased from Harlan-CPB, Zeist, the Netherlands, and were kept under routine laboratory conditions with a 12 hour light-dark cycle at the Central Animal Laboratory of the University of Groningen. The rats received standard laboratory chow and had free access to food and water. The Local Committee for Care and Use of Laboratory Animals approved this study.
Animal experiments
2-AAF/PHx-induced oval cell activation in rats
Seven days before PHx, 2-AAF pellets (70 mg/pellet over a 28-day release, 2.5 mg/d, Innovative Research Inc., Sarasota, FL) or placebo pellets were placed subcutaneously in Wistar rats. PHx was performed according to the technique of Higgins and Anderson. Sham-operated animals underwent the same treatment protocol, including manipulation of the intestine and liver, but without hepatectomy. All surgery was performed under halothane anesthesia. Nine days after surgery, livers were perfused with PBS via the portal vein, excised, cut into small pieces and snap-frozen in liquid nitrogen for RNA isolation or frozen in cold 2-methyl-butane for immunohistochemistry. Tissue was stored at -80 °C prior to use.

PHx-induced hepatocyte proliferation in rats
To define transporter expression in proliferating hepatocytes, Wistar rats were either sham operated or received PHx. At 24h post PHx, hepatocytes were isolated using a two-step collagenase perfusion as described before. Cell fractions were frozen in liquid nitrogen prior to RNA isolation.

Isolation of hepatic cell fractions
Specific hepatic cell fractions were isolated from male Fischer 344 rats. Cholangiocytes were isolated using serial counterflow elutriation and isopycnic centrifugation and further purified by immunomagnetic isolation as described by Ishii et al. Cells isolated from five rats were pooled to obtain sufficient amounts of RNA. Oval cells were isolated from rats 12 days after 2-AAF/CCl₄-treatment as described by Petersen et al. Hepatocytes were isolated as described.

Cell culture
Rat liver epithelial RLE ϕ13 cells were a kind gift from Dr. S.S. Thorgeirsson, Laboratory of Experimental Carginogenesis, Division of Basic Sciences, National Cancer Institute, Bethesda and have been described previously. The cells were maintained in Ham’s F12 (Invitrogen Life Technologies, Paisley, UK) supplemented with 10% FCS and 50 µg/mL gentamycine (Invitrogen Life Technologies). Cells were grown to 90-95% confluence prior to RNA isolation in a humidified incubator at 37°C/5% CO₂.

Antibodies
The mouse monoclonal antibody C219 (Dako, Glostrup, Denmark) was used for detection of all P-glycoproteins (Pgps). This antibody recognizes a conserved epitope close to the ATP-binding cassette in all known members of the P-glycoprotein subfamily. The goat polyclonal anti-MRP1 antibody SC-7774 (Santa Cruz Biotechnology, Santa Cruz, CA) was used for detection of Mrp1. The epitope detected by anti-MRP1 is located in the amino terminus of human MRP1 and is not conserved in other Mrp family members. The rabbit polyclonal anti-rat Mrp3 antibody was a kind gift from Dr H. Suzuki (University of Tokyo, Tokyo, Japan). OV-6 was a kind gift from Dr S. Sell (Albany Medical College, Albany, NY).
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**Immunohistochemistry**

For immunohistological studies, 4 \( \mu \)m sections were cut from frozen liver tissue. After drying, sections were fixed in acetone for 10 min at room temperature and washed in PBS, immediately before use. Primary antibodies were incubated for 30 min at room temperature. For monoclonal antibodies a peroxidase staining method was used with secondary antibodies, preabsorbed with rat serum (Sigma, St Louis, MO). For the rabbit polyclonal antibodies, the rabbit-EnVision staining method (Dako) was used. For the goat polyclonal antibody, the secondary step consisted of swine anti-goat IgG (Dako), followed by goat peroxidase-anti-peroxidase (Dako). Secondary and tertiary antibodies were diluted in PBS containing 10% normal rat serum. All incubation steps were performed for 30 min at room temperature and were followed by 3 washes in 3 changes of PBS for 5 min.

To evaluate the induction of protein expression, dilution series of the primary antibodies were used (1/10; 1/30; 1/50; 1/100; 1/200; 1/500; 1/1000). Sections from animals from different experimental groups were put together as much as possible on a single slide and different slides were stained in the same batch. When staining was at the limit of detectability and disappeared at a higher dilution, it was evaluated as “critical staining”. For each liver specimen, this critical staining was evaluated in each cell compartment separately. By comparing the dilution at which the critical staining was seen, we could evaluate by which factor the protein was up-regulated in each liver cell compartment.

**RNA isolation and quantitative PCR**

Total RNA was isolated from tissue or cells using TRIzol (Invitrogen Life Technologies) followed by the SV Total RNA isolation system (Promega, Madison, WI) according to manufacturer’s instructions. Reverse transcription was performed on up to 5 \( \mu \)g of total RNA using random primers in a final volume of 75 \( \mu \)L (Reverse Transcription System, Promega).

The cDNA levels of the various genes were measured by real-time PCR using the ABI PRISM 7700 sequence detector (Applied Biosystems, Foster City, CA), a procedure in which cDNA levels are quantitated using a fluorescence signal that is generated during the PCR amplification by cleavage of a fluorogenic probe. Cycle numbers at which the sample fluorescence signal increases above a fixed threshold level (\( C_T \) value) correlate inversely with the mRNA levels. Four microliters of diluted cDNA were used in each PCR reaction in a final volume of 20 \( \mu \)L, containing 900 nM of sense and of antisense primers, 200 nM of fluorogenic probe, 5 mM MgCl\(_2\), KCl, Tris-HCl, 0.2 mM dATP, dCTP, dGTP, dTTP, dUTP and 0.5 U of AmpliTaq DNA polymerase (qPCR Core Kit, Eurogentech, Seraing, Belgium). Sequences of the primers and probes used are listed in Table 3.1. Probes were labelled by a 5’ FAM (6-carboxy-fluorescein) reporter and a 3’ TAMRA (6-carboxytetramethyl-rhodamine) quencher. The PCR program was 95°C for 10 min, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Each sample was analysed in duplicate. For relative quantification of mRNA expression calibration curves were constructed expressing the log of the input amount as \( x \) and \( C_T \) as \( y \). 18S expression levels were used as endogenous control.
ABC transporter expression in rat liver progenitor cells

Statistics

The data resulting from the experimental groups were expressed as the means ± SD. Differences between the four experimental groups in the 2-AAF/PHx experiment were determined by one-way ANOVA analysis, with posthoc comparison by Student-Newman-Keuls test (SPSS software). An unpaired Student’s t test was used to compare the means between the two groups. A P value < 0.05 was considered significant.

3.4 Results

Characteristics of partial hepatectomized rats

Rats were treated either with 2-AAF or placebo for 7 days before undergoing a partial hepatectomy (PHx) or sham operation. The average body weight of the rats was 196 ± 13.1 g when pellets were placed and 242 ± 17.1 g at the time of surgery. There was no significant difference in body weight between rats receiving placebo pellets and rats receiving 2-AAF-containing pellets. On average 7.6 ± 0.7 g of liver were excised during PHx. Animals undergoing 2-AAF/PHx-treatment gained approximately 20 g in weight during the 9 days after PHx, compared to approximately 40 g for rats in the placebo/sham, placebo/PHx, and 2-AAF/sham groups. Liver weights from sham-operated animals were used to calculate the % liver weight/body weight (4.5%). From this, the percentage of liver removed from the partial hepatectomized rats was estimated to be 71%.

ABC transporter gene expression during oval cell activation

2-AAF-treatment without PHx resulted in a mild proliferation of oval cells (Figure 3.1A) as reflected by the presence of more ductuli than seen in normal rat liver (not shown). Massive oval cell proliferation was seen in livers of 2-AAF/PHx treated rats 9 days after PHx (Figure 3.1B).

Figure 3.3 shows the hepatic expression of ABC genes of the four experimental groups, 9 days after PHx or sham operation. The expression of Mdr1b mRNA was significantly increased both in 2-AAF and in 2-AAF/PHx-treated rats (79 ± 20 and 120 ± 53 fold relative to 1.0 ± 0.6 and 0.3 ± 0.07 in the placebo/sham and placebo/PHx group). Both Mdr1a and Bsep mRNA expressions did not significantly change during any of the treatments while Mdr2 was increased 2-fold in 2-AAF/PHx-treated animals when compared to controls. The expression of Mrp2 and Mrp6 was not affected by any treatment, whereas Mrp1 and Mrp3 were clearly induced by 2-AAF/PHx-treatment, i.e. approximately 2.5- and 10-fold, respectively. Mrp4 expression was increased 2-fold under these conditions. Abca1 and Bcrp mRNA expression levels did not change significantly after either treatment.

Immunohistochemical staining

Using immunohistochemistry, the localization of a number of ABC transporters was determined. Figure 3.4A shows the clear canalicular staining pattern of C219, recognizing all P-glycoproteins, in normal rat liver. This C219 staining pattern was much more pronounced in periportal hepatocytes in livers of 2-AAF (not shown) and 2-AAF/PHx-treated
Table 3.1: Sequences of PCR primers and probes used for real-time detection PCR analysis

<table>
<thead>
<tr>
<th>cDNA</th>
<th>Primers</th>
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</thead>
<tbody>
<tr>
<td>Mdr1a sense</td>
<td>5'-GCA GGT TGG CTG GAC AGA TT-3'</td>
</tr>
<tr>
<td>antisense</td>
<td>5'-GGA GCC CAA TTC CAT GGA TA-3'</td>
</tr>
<tr>
<td>probe</td>
<td>5' FAM-CCG CCA GAG TAA CCA GGA GAG CCT- TAMRA 3'</td>
</tr>
<tr>
<td>Mdr1b sense</td>
<td>5'-AAA CAT GCC AGC TAA CCA AAG TT-3'</td>
</tr>
<tr>
<td>antisense</td>
<td>5'-AAA ATG TGG CCC TGT TTA ATG ATT-3'</td>
</tr>
<tr>
<td>probe</td>
<td>5' FAM-CAC TGT TAA AGG TAA TTT CAT CAA GAC GAG CCT GC- TAMRA 3'</td>
</tr>
<tr>
<td>Mdr2 sense</td>
<td>5'-AGT TCA CCG GCG CAT CAA-3'</td>
</tr>
<tr>
<td>antisense</td>
<td>5'-AAA AGA CAC TGG TGG CAC GGT-3'</td>
</tr>
<tr>
<td>probe</td>
<td>5' FAM-CAT GAT TGG TTT CCA CAT CCA GC- TAMRA 3'</td>
</tr>
<tr>
<td>Bsep sense</td>
<td>5'-CCA AGC TGC CAA GGA TGC TA-3'</td>
</tr>
<tr>
<td>antisense</td>
<td>5'-CCT TCT CTA ACA AGG GTG TCA-3'</td>
</tr>
<tr>
<td>probe</td>
<td>5' FAM-CAT GGC CCT GCC GCA GC- TAMRA 3'</td>
</tr>
<tr>
<td>Mrp1 sense</td>
<td>5'-GCC ACT GCC TCA TGC CTG TT-3'</td>
</tr>
<tr>
<td>antisense</td>
<td>5'-GCA AGA CCT GAA GCC AAG ATA CA-3'</td>
</tr>
<tr>
<td>probe</td>
<td>5' FAM-ACC CAC ATT TAT AGC AGC AAG CCA GAG CC- TAMRA 3'</td>
</tr>
<tr>
<td>Mrp2 sense</td>
<td>5'-GAC GAT GTG CAT GGG GTG AT-3'</td>
</tr>
<tr>
<td>antisense</td>
<td>5'-CTT TTC ATG GCC AAG GAA GCT-3'</td>
</tr>
<tr>
<td>probe</td>
<td>5' FAM-CCC ACC ATG GAG GAA CCT GAG G- TAMRA 3'</td>
</tr>
<tr>
<td>Mrp3 sense</td>
<td>5'-TCC CAC TTT CGG GAC ACA GTA ACT-3'</td>
</tr>
<tr>
<td>antisense</td>
<td>5'-CTT AGC ATC ACT GAG GAC CCT GAA-3'</td>
</tr>
<tr>
<td>probe</td>
<td>5' FAM-CAG TGT CAT TGG GCC CTA CGG GC- TAMRA 3'</td>
</tr>
<tr>
<td>Mrp4 sense</td>
<td>5'-TCA GTG TTT GAC AGA GAC ATG TAG TG-3'</td>
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<tr>
<td>antisense</td>
<td>5'-CTT CTC CGG CAT TTT CTG TTA-3'</td>
</tr>
<tr>
<td>probe</td>
<td>5' FAM-TCA GTT CTA CTG GCA TAA TTT CAT CCA GAG G- TAMRA 3'</td>
</tr>
<tr>
<td>Mrp6 sense</td>
<td>5'-CTC TCC CAT TGG CTT TTT TGA G-3'</td>
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<tr>
<td>antisense</td>
<td>5'-GTC CAC ATC CAT TGT GCT GTT-3'</td>
</tr>
<tr>
<td>probe</td>
<td>5' FAM-TCC GGA ACC TGC TGA ACC CCT TTG C- TAMRA 3'</td>
</tr>
<tr>
<td>Abca1 sense</td>
<td>5'-CCC AGA GCC AAA AGC AGC TC-3'</td>
</tr>
<tr>
<td>antisense</td>
<td>5'-GCT CAT CAC TTT GGT CCT TG-3'</td>
</tr>
<tr>
<td>probe</td>
<td>5' FAM-AGA CTA CTC TGT CTT TCA GAC AAC ACT TGA CCA AG- TAMRA 3'</td>
</tr>
<tr>
<td>Bcrp sense</td>
<td>5'-CAG GTA CCC AAT TGT GAG GAA GA-3'</td>
</tr>
<tr>
<td>antisense</td>
<td>5'-AAT CAG GCC ATC GAT CGT TCA-3'</td>
</tr>
<tr>
<td>probe</td>
<td>5' FAM-CAT GCA AGC CAG GCC CAC ATG A- TAMRA 3'</td>
</tr>
<tr>
<td>Thy1 sense</td>
<td>5'-GCA GAT TTC CCG AGG ACA GA-3'</td>
</tr>
<tr>
<td>antisense</td>
<td>5'-GCC AGT CCA GTC GAA GGT TCT-3'</td>
</tr>
<tr>
<td>probe</td>
<td>5' FAM-CAG CTC GAC GCC TCT CCT GGT GA- TAMRA 3'</td>
</tr>
<tr>
<td>18S sense</td>
<td>5'-CAG TTA CCA CAT CCA AAG A-3'</td>
</tr>
<tr>
<td>antisense</td>
<td>5'-CCA ATT ACA GGG CCT CGA AA-3'</td>
</tr>
<tr>
<td>probe</td>
<td>5' FAM-CCG GCA AAT TAC CCA CTC CCG A- TAMRA 3'</td>
</tr>
</tbody>
</table>
Figure 3.1: Liver sections from rats exposed to 2-AAF only (A) or 2-AAF/PHx (B), obtained at day 9 after surgery. Sections were stained with the OV6 antibody (dilution 1/200). (A) In rats exposed to 2-AAF, OV6 stains the bile duct (right side) and a number of ductuli. (B) In 2-AAF/PHx-treated rats a massively increased number of ductuli is apparent from intensive OV6 staining. B=bile duct; P=portal tract; arrows=oval cell reaction

rats (Figure 3.4B). By diluting the antibody to the level of critical staining, the increase in protein expression for 2-AAF/PHx-treated rats was estimated to be more than 50-fold compared to control.

Figure 3.2: Immunohistochemical staining with OV6 (A) and C219 (B) in rat liver 9 days after 2-AAF/PHx-treatment, demonstrating that C219 does not stain the oval cell compartment recognized by OV6. Typical staining patterns of n=3-5 per group. arrows=oval cell reaction

Figure 3.4C shows that the expression of Mrp1 in control rat liver was extremely low. Its expression was increased after 2-AAF/PHx-treatment (Figure 3.4D). By diluting the antibody to the level of critical staining, this increase in protein expression was estimated to be 2 to 5-fold.
Chapter 3

The expression of Mrp3 in normal rat liver is shown in Figure 3.4E, with a weak sinusoidal staining of hepatocytes in the centrilobular zone. The bile ducts were also positive as shown in the insert in Figure 3.4E. In livers of 2-AAF/PHx-treated rats (Figure 3.4F) hepatocytes in the entire hepatic acinus became positive, with the strongest signal in perivenous hepatocytes. Mrp3 expression was not increased in cholangiocytes. There was, however, an additional staining of the oval cell compartment after 2-AAF/PHx. This signal was more intense than the signal from the bile ducts.

To further confirm that C219 staining did not localize to the oval cells, staining patterns of OV6 and C219 were compared in serial 4 µm liver sections from 2-AAF/PHx-treated rats. As can be seen in Figure 3.2, no significant co-localization of OV6 (Figure 3.2A) and C219 (Figure 3.2B) was observed.

**ABC transporter gene expression in isolated cell fractions**

To quantify ABC transporter gene expression in separate cell types, isolated liver cell fractions were analyzed. Thy-1 positive cells were isolated from 2-AAF/CCl₄-treated rats, 12 days after CCl₄ exposure. These cells were compared with cholangiocytes and hepatocytes isolated from normal rats and with the rat liver epithelial cell line RLE φ13. Figure 3.5 shows that Thy-1 positive cells and RLE φ13 cells, both “prototypes” of hepatic progenitor cells, resemble cholangiocytes with respect to ABC transporter gene expression. While normal hepatocytes highly expressed Mdr2, Bsep, Mrp2, and Mrp6 mRNA, these transporters were virtually absent in Thy-1 positive cells, RLE φ13 cells, and cholangiocytes. However, Thy-1 positive cells and hepatocytes, but not cholangiocytes, expressed high levels of Abca1 mRNA. Comparison of transporter expression in RLE φ13 with that of Thy-1 positive cells showed that RLE φ13 cells have similar expression characteristics as Thy1-positive cells. The exception in this respect was Mdr1b mRNA, which was over-expressed in the RLE φ13 cell line.

**ABC transporter gene expression in proliferating hepatocytes**

For comparison we also measured the ABC transporter gene expression in proliferating hepatocytes, isolated 24 h after PHx. This time point was chosen because a peak in DNA synthesis, as measured by BrdU incorporation, was observed 24 h after PHx (data not shown). These results are summarized in Table 3.2. Proliferating hepatocytes had a high expression of Mdr1b mRNA. The levels of Mdr2, Mrp1, and Mrp4 were slightly increased. However, Mrp3 expression remained unchanged.

### 3.5 Discussion

In this study we demonstrate that the hepatic mRNA expression of Mdr1b, Mrp1, and Mrp3 transporter genes is induced in the 2-AAF/PHx rat model of oval cell proliferation. Immunohistochemistry on livers of 2-AAF/PHx-treated rats showed high expression of Mrp1 and Mrp3 in oval cells. In addition, we show that isolated Thy-1 positive cells and cultured RLE φ13 cells, prototypes of hepatic progenitor cells, both express particular high levels of Mrp3 and Mrp1. This supports the in vivo results of the 2-AAF/PHx model. High
ABC transporter expression in rat liver progenitor cells

Figure 3.3: mRNA levels of ABC transporter genes in the liver during oval cell activation by 2-AAF/PHx-treatment. Relative mRNA expression levels of various ABC transporter genes were determined by real time detection RT-PCR as described in Materials and methods. plac/sham: placebo pellet followed by sham operation; plac/PHx: placebo pellet followed by PHx; 2-AAF/sham: 2-AAF pellet followed by sham operation; 2-AAF/PHx: 2-AAF pellet followed by PHx. Levels expressed relative to plac./sham levels (set to 1). Data points represent means ± S.D., n=3-5 per group, *P < 0.05.

expression of these transporters suggests that they have a role in cellular protection of this important cell population.

In the model used in this study, proliferation of hepatocytes after PHx was blocked by pre-treatment with 2-AAF. In hepatocytes, 2-AAF is converted via phase I drug metabolism. As 2-AAF or its metabolites may by themselves induce the expression of ABC transporters, one group of animals received 2-AAF alone. These animals showed a mild activation of the oval cell compartment. In vitro, 2-AAF has been shown to increase the expression of Mdr1b and Mrp2 in cultured rat hepatocytes and in rat hepatoma cell lines as well as the expression of MRP2, MRP3, and MRP5 in the human hepatoma cell line HepG2. In vivo in rats, 2-AAF has been reported to induce hepatic Mdr1b expression. Our results confirmed the large increase in Mdr1b mRNA expression but the mRNA expression levels of both Mrp2 and Mrp3 were found to be not affected by 2-AAF in the in vivo situation.
Figure 3.4: Immunohistochemical staining of C219 (A, B), Mrp1 (C, D), and Mrp3 (E, F) in control rat liver (A, C, E) and 2-AAF/PHx-treated rat liver (day 9) (B, D, F). Frozen liver sections were stained with primary antibodies directed against all P-glycoproteins (using C219, dilution 1/5), Mrp1 (SC-7774, dilution 1/5), or Mrp3 (anti-Mrp3, dilution 1/500). In normal liver, C219 weakly stained the canalicular membrane of hepatocytes (A). In treated rat liver C219 staining was increased (B). SC-7774 did not stain normal liver, but co-localized with the oval cell compartment in treated liver (D). In normal liver anti-Mrp3 stained the pericentral hepatocytes (E) and the bile ducts (insert in E). In treated there was additional staining of the oval cells (F). Typical staining patterns of n=3-5 per group. B=bile duct, P=portal tract, C=central vein; arrows=oval cell reaction.
ABC transporter expression in rat liver progenitor cells

Deng et al.\textsuperscript{50} have recently elucidated the intracellular signal transduction pathway involved in the up-regulation of Mdr1b mRNA levels by 2-AAF in an \textit{in vitro} model. They demonstrated that transcription of Mdr1b is induced by NF-κB. The activation of NF-κB is the ultimate result of oxidative stress generated by 2-AAF.\textsuperscript{50} Moreover, 2-AAF has been demonstrated to increase p53 expression.\textsuperscript{2} p53, in turn, can induce Mdr1b expression.\textsuperscript{51} The strong C219 staining pattern in rats treated with 2-AAF only can thus, at least in part, be explained by direct effects of 2-AAF on hepatocytes. After 2-AAF/PHx-treatment, when there is extensive oval cell activation, the Mdr1b protein expression seemed to be confined to periportal hepatocytes and did neither occur in the OV-6 positive cell compartment nor in bile duct epithelial cells. This observation contrasts with the human situation. In regeneration after submassive necrosis, there is increased expression of MDR1 both in the remaining hepatocytes and in the regenerating ductules, the latter representing the putative progenitor cells (Ros \textit{et al.}, submitted).

We used the commercially available antibody SC-7774 to study Mrp1 expression. This
Table 3.2: Expression of ABC transporter genes after 70% PHx relative to control as determined by real-time detection PCR analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>24 h Sham</th>
<th>24 h PHx</th>
</tr>
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<tbody>
<tr>
<td>Mdr1a</td>
<td>1.00 ± 0.37</td>
<td>1.29 ± 0.26</td>
</tr>
<tr>
<td>Mdr1b</td>
<td>1.00 ± 0.25</td>
<td>39.47 ± 1.09*</td>
</tr>
<tr>
<td>Mdr2</td>
<td>1.00 ± 0.29</td>
<td>2.08 ± 0.36*</td>
</tr>
<tr>
<td>Bsep</td>
<td>1.00 ± 0.29</td>
<td>0.89 ± 0.26</td>
</tr>
<tr>
<td>Mrp1</td>
<td>1.00 ± 0.41</td>
<td>1.90 ± 0.20*</td>
</tr>
<tr>
<td>Mrp2</td>
<td>1.00 ± 0.41</td>
<td>0.84 ± 0.08</td>
</tr>
<tr>
<td>Mrp3</td>
<td>1.00 ± 0.27</td>
<td>1.33 ± 0.39</td>
</tr>
<tr>
<td>Mrp4</td>
<td>1.00 ± 0.13</td>
<td>2.15 ± 0.35*</td>
</tr>
<tr>
<td>Mrp6</td>
<td>1.00 ± 0.21</td>
<td>0.66 ± 0.36</td>
</tr>
<tr>
<td>Abca1</td>
<td>1.00 ± 0.40</td>
<td>1.37 ± 0.23</td>
</tr>
<tr>
<td>Bcrp</td>
<td>1.00 ± 0.65</td>
<td>0.69 ± 0.13</td>
</tr>
</tbody>
</table>

antibody has been raised against an Mrp1-specific epitope. In normal liver, SC-7774 staining was extremely weak indicating that SC-7774 does not recognize Mrp2 (located in the canaliculi) or Mrp3 (located in the cholangiocytes). Furthermore, the antibody does not recognize Mrp4, as Mrp4 lacks the extra N-terminal region against which SC-7774 has been raised. Moreover, the extremely low staining in normal liver suggests that SC-7774 does not recognize Mrp6, which is highly expressed in normal liver. Thus, we conclude that SC-7774 is specific for Mrp1. The increase in SC-7774 staining observed in 2-AAF/PHx-treated rats (Figure 3.4D) therefore demonstrates increased Mrp1 expression.

To further define the expression profiles of ABC transporters in different cell types, we compared isolated liver cell fractions. We also compared Thy-1 positive cells with the liver epithelial cell line RLE $\varphi$13, which resembles oval cells, by expressing factors as c-kit, OV-6 and $\alpha$-fetoprotein. Compared to Thy-1 positive cells, RLE $\varphi$13 cells were found to have a highly increased expression of Mdr1b, a phenomenon that is frequently observed with cultured cells. Levels of other ABC transporters also change during culture. When comparing freshly isolated cells with cultured cells (hepatocytes with cultured hepatocytes and rat hepatoma cells [H4IIE cell line]; Thy-1 positive cells with cultured RLE $\varphi$13 cells; and freshly isolated cholangiocytes with cultured NRC cells) we consistently observed an increase in Mdr1a, Mdr1b and Mrp1 mRNA expression during culture. Conversely, expression levels of the hepatocellular transporters Mdr2, Bsep, Mrp2, and Mrp6 decreased during culture (own observations, as well as 54, 55). Mrp3 expression increased in most cultured cells, but the already high Mrp3 expression of Thy-1 positive cells was not further increased in RLE $\varphi$13 cells. Despite these conditional changes, the expression pattern of ABC transporters in Thy-1 positive cells is largely retained in RLE $\varphi$13 cells in that they both have high mRNA levels of Mrp1 and Mrp3 and low mRNA levels of Mdr2, Bsep, Mrp2, and Mrp6.

There was a remarkable difference between Thy-1 positive cells and cholangiocytes in expression of Abca1, which was found to be expressed in Thy-1 positive cells but not
in cholangiocytes. The function of Abca1 in oval cells is not yet known. It may promote engulfment of apoptotic cells by translocating phosphatidylserine to the outer plasma membrane leaflet, which would correlate with the high prevalence of apoptosis in the oval cells compartment.

It has recently been shown that primitive hematopoietic stem cells highly express Bcrp. As hepatic oval cells display primitive features, we speculated that these cells also have a high Bcrp expression. However, in the 2-AAF/PHx model, Bcrp mRNA expression was not induced. Moreover, Bcrp mRNA expression levels were similar in the different cell fractions studied. It may be that Thy-1 positive cells have already acquired a more differentiated phenotype and have lost Bcrp expression.

This study demonstrates that hepatic oval cells have high expression levels of Mrp1 and Mrp3. Proliferating hepatocytes, isolated 24 h after PHx, expressed highly increased levels of Mdr1b whereas Mdr2, Mrp1, and Mrp4 were increased to a lesser extent (Table 3.2). Mrp3 expression was not increased in proliferating hepatocytes after PHx. Thus, Mrp3 up-regulation mainly occurs in oval cells, whereas Mdr1b up-regulation mainly takes place in hepatocytes.

In conclusion, our findings show that the up-regulation of Mdr1b in the 2-AAF/PHx model is confined to hepatocytes while the expression of Mrp1 and Mrp3 mainly occurs in the oval cell compartment. Hepatic oval cells and cholangiocytes bear a close resemblance as far as their ABC transporter expression is concerned. Both cell types appear to be well protected by ABC transporters, which may enable these cells to survive conditions associated with excessive metabolic stress and serve as a proliferative reservoir when hepatocytes are severely damaged.

3.6 Acknowledgment

The authors thank Dr S.S. Thorgeirsson, for providing the RLE ϕ13 cell line and Dr H. Suzuki and Dr S. Sell for proving the Mrp3 and OV6 antibodies. This study was supported by grant NWO 902-23-191 of the Netherlands Organization for Scientific Research.

3.7 References


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