Expression and regulation of ABC transporter genes during liver regeneration
Ros, Jenny

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Chapter 4

High expression of MDR1, MRP1, and MRP3 in the hepatic progenitor cell compartment and hepatocytes in severe human liver disease

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Submitted
4.1 Abstract

In diverse human liver diseases an increase in bile ductular structures is observed. These structures harbour the progenitor cell compartment of the liver. Since ATP-binding cassette transporters may serve a cytoprotective role in liver disease, we performed an immunohistochemical study on human liver specimens of patients with submassive cell necrosis, chronic hepatitis C (HCV), primary biliary cirrhosis (PBC), and normal liver. The expressions of MDR1, MDR3, BSEP, MRP1, MRP2, and MRP3 were determined using specific antibodies. Dilution series were made until the critical staining level, to semiquantify the factor of up-regulation. In normal liver, hepatocytes showed a canalicular staining for MDR3, BSEP, and MRP2. MDR1 stained the canalicular membrane of the hepatocytes as well as of the cholangiocytes. MRP3 showed low immunoreactivity of the bile duct epithelial cells and of the pericentral hepatocytes only. Normal liver was not immunoreactive for MRP1. In diseased liver, the expression of MDR3, BSEP, and MRP2 was relatively stable. Only during severe cholestasis, the expression of BSEP and MRP2 decreased. The expression levels of MDR1, MRP1, and MRP3, however, were increased in PBC, HCV, or submassive necrosis. Strongest immunoreactivity was seen after submassive necrosis, where remaining islands of hepatocytes showed strong canalicular staining for MDR1 (20-50-fold up-regulation) and MRP3 (20-fold up-regulation). The expression of MRP1 in remaining hepatocytes was heterogeneous. Regenerating bile ductules at the interface of portal tracts and necrotic areas intensely stained for MDR1, MRP1, and MRP3. In conclusion, MDR1, MRP1, and MRP3 are up-regulated in hepatocytes in severe human liver disease. Strong MDR1-, MRP1-, and MRP3-reactivity is seen in human regenerating bile ductules.
4.2 Introduction

In various human and rodent liver diseases a striking increase in the number of bile ductular structures is observed. These form a labyrinth at the edge of the portal tracts (ductular reaction). These structures harbour the progenitor cell compartment of the liver.\(^1\)

Hepatic progenitor cells are activated after liver damage and have a critical role in hepatic repair after excessive damage.\(^1\)–\(^4\) We therefore hypothesize that these 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.

In normal liver ABC transporters participate in diverse cellular processes, mediating excretion of bile salts, organic anions, lipids or xenobiotics. This way they contribute to bile formation and enable cells to keep the intracellular levels of toxic compounds low.

The ABC transporter family has been divided in 7 subclasses (A-G) (reviewed by Klein \textit{et al.}\(^5\) and Borst and Oude Elferink\(^6\)). Members of the ABCB and ABCC subfamilies are important for bile formation. Members of the ABCB subfamily that are expressed in the liver include MDR1 (gene symbol \textit{ABCB1}), an efflux pump for hydrophobic compounds, MDR3 (\textit{ABCB4}), a translocator of phosphatidylcholine across the membrane, and BSEP (\textit{ABCB11}), the export pump for bile salts. These transporters are all located in the apical membrane of hepatocytes.

The ABCC subfamily consists of 12 members, of which five have now been identified in liver tissue. Best characterised are the apical multidrug resistance related protein 2 (MRP2, \textit{ABCC2}) and its basolateral homologues MRP1 (\textit{ABCC1}) and MRP3 (\textit{ABCC3}). MRP2 transports amphiphilic anionic conjugates into bile. MRP1 transports the same substrates, but its expression levels are very low in normal liver. MRP3 is able to transport organic anions, including bile salts, into blood.\(^7\),\(^8\) Expression levels of MRP2 and MRP3 appear to be counter-regulated: when MRP2 levels decrease, an increase in MRP3 expression is observed.\(^9\),\(^10\) This provides the cell with a compensatory mechanism for secretion of bile salts under cholestatic conditions. In addition to its expression in hepatocytes, MRP3 has been identified in the basolateral membrane of cholangiocytes.\(^11\)

We have previously studied ABC transporter gene expression in a rat model of hepatic progenitor cell activation. These rats were treated with 2-acetylaminofluorene (2-AAF) prior to partial hepatectomy. We observed a high expression of Mrp1 and Mrp3 in the progenitor cells in these rats (Ros \textit{et al.}, submitted).

Here we aimed to study ABC transporter gene expression in human liver with progenitor cell activation. To study the different human liver cell compartments separately, we performed immunohistochemical analyses using specific antibodies against ABC transporter molecules. To evaluate up- or down-regulation of the transporter proteins in the different segments of the bile duct system and the hepatocytes, we made dilution series of the primary antibodies until the staining disappeared in the different cell compartments.
4.3 Materials and methods

**Immunohistochemistry**

Forty human liver specimens taken from liver explants prior to liver transplantation or needle biopsies taken for diagnostic purposes, were used for this study: normal liver (n=5), PBC (n=10), HCV (n=15) and regeneration after submassive liver necrosis (n=10). From each specimen, part was snap-frozen in liquid nitrogen-cooled isopentane, part was fixed in B5 fixative or in formaldehyde and embedded in paraffin for routine diagnosis, and part was fixed for electron microscopy. The diagnosis was made on the basis of examination of the paraffin-embedded material, clinical and laboratory data. Informed consent was obtained from all patients and the experimental protocol was approved by the Ethical Committee of the University Hospital of Leuven, in accordance with the Ethical Guidelines of the 1975 Declaration of Helsinki.

Normal liver biopsies showed a preserved architecture, no inflammation, no steatosis, and no cholestasis. Biopsies from patients with PBC showed advanced portal-portal septum formation in 8 patients and established cirrhosis in 2 patients. There was advanced ductopenia and a marked ductular reaction at the periphery of the portal tracts and septa. A marked degree of portal inflammation and granuloma formation was seen. Periportal and perisepal hepatocytes showed signs of cholate stasis with swelling and a pale aspect of the cytoplasm, sometimes with formation of cholestatic Mallory bodies. Cholestatic liver cell rosettes and bilirubinostasis were also present.

Biopsies of chronic HCV patients showed a varying degree of fibrosis ranging from periportal fibrosis (n=5) over septal fibrosis (n=4) to cirrhosis (n=6). The portal tracts showed varying degrees of mononuclear inflammatory infiltrate with lymphoid aggregate formation and interface hepatitis. Also lobular/nodular inflammation was variable.

In specimens with submassive liver cell necrosis, large areas of parenchyma had disappeared, leaving only islands of remaining hepatocytes. High numbers of ceroid macrophages were seen. At the edges of portal tracts, regenerating ductules were present. The degree of infiltrate in the portal tracts and necrotic areas varied according to the cause of necrosis. In the cases of toxic liver cell necrosis the infiltrate was moderately dense and contained a relatively high number of eosinophilic polymorphonuclear leucocytes. In the cases of viral hepatitis, the lymphocytic infiltrate was very marked. In the cases of auto-immune hepatitis, lymphocytes were intermingled with large clusters of plasma cells.

Immunohistochemistry was performed on frozen sections. Five micrometers thick cryostat sections were dried overnight, were subsequently fixed in acetone for 10 minutes and finally washed in PBS, immediately before use. All samples were incubated with the primary antibody for 30 min at room temperature. The primary antibodies used are listed in Table 4.1. P3II-26, M2II-4 and M3II-9 were kindly provided by Dr. R.J. Schepfer, Free University, Amsterdam, the Netherlands. JSB1 was obtained from Alexis Biochemicals (Lausen, Switzerland). SC-7774 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). OV-6 was a kind gift from Dr. S. Sell (Albany Medical College, Albany, NY). Anticytokeratin 7 and 19 were obtained from Dako (Glostrup, Denmark) and from Amersham Life Sciences (Amersham, UK), respectively. With all rabbit polyclonal antibodies, a two-step
Table 4.1: List of antibodies used

<table>
<thead>
<tr>
<th>name</th>
<th>species</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDR1</td>
<td>JSB1</td>
<td>mouse mAB</td>
</tr>
<tr>
<td>MDR3</td>
<td>P3II-26</td>
<td>mouse mAB</td>
</tr>
<tr>
<td>BSEP</td>
<td>k12</td>
<td>rabbit pAB</td>
</tr>
<tr>
<td>MRP1</td>
<td>SC 7774</td>
<td>goat pAB</td>
</tr>
<tr>
<td>MRP2</td>
<td>M2I-4</td>
<td>mouse mAB</td>
</tr>
<tr>
<td>MRP3</td>
<td>M3II-9</td>
<td>mouse mAB</td>
</tr>
</tbody>
</table>

Peroxidase-labeled method was used. Slides were blocked with normal goat serum (dilution 1/5 in PBS) for 7 min, followed by incubation with the primary antibody for 30 min at room temperature. Subsequently, slides were incubated with undiluted Rabbit-EnVision (Dako) for 30 min at room temperature. With the goat polyclonal antibodies, the second step consisted of swine anti-goat IgG (Dako), followed by goat peroxidase-anti-peroxidase (Dako). With all monoclonal antibodies, a three-step indirect immunoperoxidase procedure was used: a peroxidase-labeled rabbit anti-mouse IgG (Dako) was followed by peroxidase-labeled swine anti-rabbit IgG (Dako). All secondary and tertiary antibodies were diluted in PBS containing 10% normal human serum. All incubation steps were followed by a wash in three changes of PBS for 5 min. The reaction product was developed with the use of 3-amino-9-ethylcarbazole and H₂O₂ (0.01%) and finally the sections were counterstained with haematoxylin. Controls consisted of omission of the primary antibody or replacement by normal rabbit immunoglobulins (Dako) or normal mouse ascites (Dako) and showed absence of specific staining.

To evaluate the factor up-regulation of the proteins, dilution series of the primary antibodies were used (1/5; 1/10; 1/20; 1/30; 1/50; 1/100; 1/200; 1/300; 1/500; 1/1000). Four to five frozen sections were put together on one slide in order to stain all sections in the same conditions. When a staining was at the limit of detectability and disappeared in a higher dilution, we evaluated this as “critical staining”. For each liver specimen this critical staining was evaluated in each cell compartment separately (hepatocytes, ductules, interlobular ducts). By comparing the dilution at which the critical staining was seen, we could evaluate with which factor the protein was up-regulated in each liver cell compartment. Evaluations of the stainings were done by two independent pathologists in a blinded way. The slides were mixed up randomly and coded by an independent person.

**RNA isolation and reverse-transcriptase polymerase chain reaction (RT-PCR)**

Total RNA was isolated from tissue using TRIzol (Invitrogen Life Technologies, Breda, the Netherlands) according to manufacturers instructions. Reverse transcription was performed on 3.5 µg of total RNA using random primers in a final volume of 75 µL (Reverse Transcription System, Promega, Madison, WI).

The cDNA levels of the various genes were measured by real-time polymerase chain reaction (PCR) using the ABI PRISM 7700 sequence detector (Applied Biosystems, Foster City, CA) where cDNA levels are quantitated using a fluorescence signal that is generated
Table 4.2: Sequences of PCR primers and probes used for real-time detection PCR analysis

<table>
<thead>
<tr>
<th>cDNA</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDR1</td>
<td>sense: 5'-GGC AAA GAA ATA AAG CGA CTG AA-3'</td>
</tr>
<tr>
<td></td>
<td>antisense: 5'-GGC TGT TGT CTC CAT AGG CAA T-3'</td>
</tr>
<tr>
<td></td>
<td>probe: FAM-CCT GTC CCA GGA GCC CAT CCT GT TAMRA 3'</td>
</tr>
<tr>
<td>MDR3</td>
<td>sense: 5'-TCA ATG GCT TTT AAA GCA ATG CTA-3'</td>
</tr>
<tr>
<td></td>
<td>antisense: 5'-TGC AAT TAA AGC CAA CCT GGT T-3'</td>
</tr>
<tr>
<td></td>
<td>probe: FAM-CAC AGA TGG CCA AGT CCA AGG A TAMRA 3'</td>
</tr>
<tr>
<td>BSEP</td>
<td>sense: 5'-ACA TGC TTG CGA GGA CCT TTA-3'</td>
</tr>
<tr>
<td></td>
<td>antisense: 5'-GGA GGT TCG TGC ACC AGG TA-3'</td>
</tr>
<tr>
<td></td>
<td>probe: FAM-CCA TCC GGC AAC GCT CCA AGT CT TAMRA 3'</td>
</tr>
<tr>
<td>MRP1</td>
<td>sense: 5'-CTT CTT GAG GAA TTT GCT GGA TTA-3'</td>
</tr>
<tr>
<td></td>
<td>antisense: 5'-GCT AGA CCC AGA CAA GGA TGT TAG A-3'</td>
</tr>
<tr>
<td></td>
<td>probe: FAM-TCT TTT AGA TGC TTT TGG CTA CCA CTG CCA C TAMRA 3'</td>
</tr>
<tr>
<td>MRP2</td>
<td>sense: 5'-TGC AGC CTC CAT AAC CAT GAG-3'</td>
</tr>
<tr>
<td></td>
<td>antisense: 5'-CTT CGT CTT CCT TCA GGC TAT TCA-3'</td>
</tr>
<tr>
<td></td>
<td>probe: FAM-CAG CTT TCG TCG AAC ACT TAG CCG ACA TAMRA 3'</td>
</tr>
<tr>
<td>MRP3</td>
<td>sense: 5'-GCC ATC GAC CTG GAG ACT GA-3'</td>
</tr>
<tr>
<td></td>
<td>antisense: 5'-GAC CCT GGT GGT CAT CAT AGT G-3'</td>
</tr>
<tr>
<td></td>
<td>probe: FAM-CAT CCG CAC CCA GGT TGA TAC CTG CAC TAMRA 3'</td>
</tr>
<tr>
<td>18S</td>
<td>sense: 5'-CGG CTA CCA CAT CCA AGG A-3'</td>
</tr>
<tr>
<td></td>
<td>antisense: 5'-CCA ATT ACA GGG CCT CGA AA-3'</td>
</tr>
<tr>
<td></td>
<td>probe: FAM-CGC GCA AAT TAC CCA CTG CCG A TAMRA 3'</td>
</tr>
</tbody>
</table>

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.\(^{12}\) Four microliters of diluted cDNA were used in each PCR reaction in a final volume of 20 µL, 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 4.2. Probes were labelled by a 5' FAM (6-carboxy-fluorescein) reporter and a 3' TAMRA (6-carboxy-tetramethyl-rhodamine) quencher. The PCR program was 95°C for 10 minutes, followed by 40 cycles of 15 seconds at 95°C and 1 minute 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.
4.4 Results

Enhanced expression of MDR1, MRP1, and MRP3 protein in reactive bile ductules and hepatocytes

In normal liver, MDR1 showed a canalicular staining pattern in hepatocytes. At a dilution of 1/10, this hepatocyte canalicular staining was at the “critical staining” level. The interlobular bile ducts showed apical reactivity, which was at the critical staining level at a dilution of 1/30 (Figure 4.1A, Table 4.3). In advanced PBC, MDR1 showed strong canalicular staining at a dilution of 1/10. The critical staining level was 1/50. This means a factor 5 up-regulation compared to normal. Reactive ductules, identified by their immunoreactivity for OV-6, cytokeratin 7 and 19, were strongly immunoreactive at the apical pole at a 1/10 dilution with a critical staining level of 1/50. In chronic HCV, canalicular staining of MDR1 was up-regulated with a factor 20-30 (critical staining at a dilution of 1/200-1/300). The stronger up-regulation was seen in severely active hepatitis, as well in early stages of fibrosis as in the cirrhotic stage. Reactive ductules showed very strong apical reactivity, with a critical staining of 1/300 to 1/1000, the strongest up-regulation seen in liver biopsies with severely active hepatitis (Figure 4.1B). In regeneration after submassive necrosis up-regulation of canalicular reactivity of MDR1 with a factor 20-50 was seen in islands of remaining hepatocytes, while regenerating ductules/progenitor cells showed strong reactivity which was also still recognizable at dilution 1/200-1/500 (Figures 4.1C, D).

MRP1 could not be detected in normal human liver. In advanced PBC, chronic HCV, and submassive necrosis, hepatocytes were heterogeneously stained at a dilution of 1/5-1/10. Some interlobular bile ducts were weakly positive at a dilution of 1/5, while some were negative at this dilution. In severely active hepatitis as well as in regeneration after submassive necrosis, reactive ductules showed strong reactivity, with a critical staining level at a dilution of 1/10 (Figure 4.1H).

In normal human liver, MRP3 showed basolateral hepatocyte reactivity in 2-3 layers of hepatocytes surrounding the central vein and in the interlobular bile ducts at a dilution of 1/10. This staining had already disappeared at a dilution of 1/30 (Figure 4.1E). In advanced PBC, basolateral hepatocytic staining in the centrolobular zone was still present at a dilution of 1/100. In addition, periportal hepatocytes were reactive. In cirrhotic end-stage PBC, diffuse nodular hepatocyte staining was present at a dilution of 1/10, 1/30 and 1/50, but disappeared at dilution 1/100. Basolateral ductular reactivity in the majority of bile ductules was still visible at dilution 1/100. Remaining interlobular bile ducts were negative at this dilution. This means an overall up-regulation with a factor 10 in centrolobular, and periportal hepatocytes and in reactive bile ductules in advanced PBC, while interlobular bile ducts showed no (or less) up-regulation (Figure 4.1F). In chronic HCV, the degree of MRP3 up-regulation was less pronounced in specimens with little inflammatory activity. In liver specimens with severely active hepatitis, basolateral hepatocyte reactivity was seen throughout the lobule/cirrhotic nodule. The up-regulation varied from a factor 2 to 20, depending on the degree of inflammation. Regenerating ductules showed strong reactivity at a dilution of 1/10, and critical staining at a dilution of 1/50. This means a
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Table 4.3: Overview of antibody dilutions for critical staining for MDR1 and MRP3

<table>
<thead>
<tr>
<th></th>
<th>normal liver</th>
<th>advanced PBC</th>
<th>chronic hepatitis C</th>
<th>submassive necrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>hepatocyte</td>
<td>ILBD</td>
<td>hepatocyte</td>
<td>ductules</td>
</tr>
<tr>
<td>MDR1</td>
<td>1/10</td>
<td>1/30</td>
<td>1/50</td>
<td>1/200-1/300</td>
</tr>
<tr>
<td>MRP1</td>
<td>n.d.</td>
<td>n.d.</td>
<td>1/5-1/10</td>
<td>1/5-1/10</td>
</tr>
<tr>
<td>MRP3 CL</td>
<td>1/10</td>
<td>1/10</td>
<td>CL</td>
<td>1/100</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ILBD: Interlobular bile ducts, n.d.: not detectable, CL centrolobular

factor 5 up-regulation. In regeneration after submassive necrosis remaining islands of hepatocytes showed diffuse basolateral reactivity for MRP3, up-regulated with a factor 20. Regenerating ductules showed strong reactivity at dilution 1/10 and critical staining at dilution 1/50 and for a subset of ductules at dilution 1/100 (Figure 4.1G). The results of these dilution series are summarized in Table 4.3.

**MDR3, BSEP, and MRP2 expression remains relatively stable during hepatic disease**

MDR3, BSEP, and MRP2 showed a canalicular staining pattern of hepatocytes, while bile ducts and ductules were negative. No clear differences in expression were seen between normal liver, PBC, chronic hepatitis, or submassive necrosis. Only in end-stage PBC, scattered hepatocytes with extreme cholate stasis, containing cholestatic Mallory bodies, showed down-regulation of MRP2 and to a lesser extent of BSEP.

**RT-PCR**

To strengthen the results obtained by immunohistochemistry, we performed quantitative RT-PCR to determine mRNA expression levels of a number of transporters. RNA was isolated from liver biopsies, and only from a limited number of samples sufficient RNA was obtained (4 controls, 3 submassive necrosis, and 3 HCV patients). These results are shown in Figure 4.2. Although levels of transporters were highly variable among the samples, and the groups are small, some trends could be observed. In total liver, MDR1 expression levels had a tendency to increase. Likewise, MRP1 and MRP3 levels increased to some extent. BSEP and MRP2 mRNA expression was relatively stable, with one exception: one patient with HCV had a high BSEP mRNA expression.

**4.5 Discussion**

Since ABC transporters play both a secretory and a protective role, we studied the expression and regulation of ABC transporters in different human liver cell compartments (hepatocytes, ductular/progenitor cell compartment, interlobular bile ducts) in normal and diseased human liver, using immunohistochemistry and dilution series. The results were further strengthened by RT-PCR.

In normal human liver, MRP3 was expressed in 2-3 layers of centrolobular hepatocytes, as is seen in the rat.10,13 The function of this export pump could be the centrolobular secretion of glucuronides into the circulation, since these are predominantly formed in the
pericentral hepatocytes. In chronic cholestasis (PBC), periportal hepatocytes became immunoreactive for MRP3. In the cirrhotic stage, some nodules showed diffuse MRP3 reactivity, in accordance to what is seen after long-term bile duct ligation in the rat. This up-regulation probably serves as a protective mechanism against the accumulation of toxic bile salts in hepatocytes. In contrast, the canalicular organic anion transporter MRP2 showed a down-regulation in severely cholestatic hepatocytes, while BSEP expression was decreased in severe cholestatic hepatocytes in some patients, but remained stable in other patients. The fact that these canalicular transporters were only down-regulated in hepatocytes with severe cholestasis, containing cholestatic Mallory bodies, suggests that these transporters are maintained in their location and perform their function for a long time, even in cholestatic livers. MRP2 and MRP3 have overlapping substrate specificity, so the apical down-regulation of MRP2 and the basolateral up-regulation of MRP3 in periportal cholestatic hepatocytes probably represents an adaptation mechanism which protects hepatocytes against accumulation of toxic bile constituents.

MDR1 and MRP3 were up-regulated in reactive bile ductules/progenitor cells. In chronic cholestatic liver diseases, reactive ductules form a labyrinth at the edge of the portal tracts and are thought to form a reservoir in which toxic bile can accumulate. The cholangiocellular expression of MDR1 and MRP3 in normal human liver specimens was stronger than the canalicular MDR1 and basolateral MRP3 expression in hepatocytes. MDR1 and MRP3 were both up-regulated in reactive bile ductules/progenitor cells.

In chronic cholestatic liver diseases, reactive ductules form a labyrinth at the edge of the portal tracts and are thought to form a reservoir in which toxic bile can accumulate. The up-regulation of MDR1 and MRP3 in ductular structures in PBC probably serves as a protective measure against the accumulation of toxic bile constituents. The fact that reactive bile ductules close to the parenchyma showed higher reactivity for MRP3 compared to ductules deeper in the portal tract and interlobular bile ducts supports this hypothesis. MRP3 probably functions as basolateral transporter to extrude bile salts back to the systemic circulation, thereby contributing to cholehepatic shunting.

In conditions of hepatocyte injury and loss, like in hepatitis, ductular reaction contributes to hepatocyte regeneration. Reactive bile ductules are at least partly the result of activation, proliferation, and differentiation of bipotential progenitor cells, which can differentiate towards hepatocytes and bile duct epithelial cells. The striking up-regulation (factor 20-50) of MDR1 and to a lesser extent of MRP3 (factor 5 in regenerating ductules, factor 2-20 in hepatocytes) and MRP1 in this cell compartment and in remaining hepatocytes could provide these cells with a multidrug-resistant phenotype. It has been demonstrated that MDR1 has anti-apoptotic effects. Via MRP1, cells can secrete products of oxidative stress reactions, such as GSSG or the GSH conjugates of 4-hydroxynonenal. This could be especially relevant for the progenitor cell compartment and the remaining islands of hepatocytes. The up-regulation of MDR1, MRP1, and MRP3 was seen in viral hepatitis and in submassive liver cell necrosis of different etiologies, suggesting that this may be a general protective mechanism, rather than a toxin-induced effect.

We aimed to quantify the mRNA levels of relevant ABC transporters using RT-PCR. mRNA levels of ABC transporter genes in total liver were quantitated using 18S as an internal standard. This quantification is complicated by the presence of additional cells
Figure 4.1: Immunohistological staining of MDR1 (A-D), MRP1 (H), and MRP3 (E-G) in human liver biopsies (A) MDR1 staining of normal liver, showing weak immunoreactivity of the parenchymal cells and more intense staining of the interlobular bile duct (arrow)(dilution 1/10). (B) MDR1 expression in severely active HCV, showing very strong apical reactivity of the ductules (arrows) and canalicular staining of the hepatocytes (arrow heads)(dilution 1/50, critical dilution was seen at dilution 1/200). (C) MDR1 expression in submassive necrosis, showing strong immunoreactivity of the regenerating ductules (arrows)(dilution 1/50). (D) MDR1 expression in submassive necrosis, showing increased immunoreactivity of the remaining hepatocytes (arrows)(dilution 1/50). (E) MRP3 expression in normal liver, showing weak immunoreactivity of the pericentral hepatocytes (dilution 1/10). C: central vein. (F) MRP3 expression in PBC, showing basolateral immunoreactivity of the periportal hepatocytes (arrows) and ductules (arrow heads). The interlobular bile ducts (large arrow) stain negative at this dilution, underlining the high expression of MRP3 in the ductules (arrows)(dilution 1/100). (G) MRP3 expression in submassive necrosis, showing the strong immunoreactivity of the ductules (arrows) and a honeycomb-pattern of hepatocytes (dilution 1/100). (H) MRP1 expression in severely active HCV showing positivity in reactive ductules (arrows) (dilution 1/5). N: necrotic area, P: portal tract

in severely diseased liver, where fibroblasts in fibrotic septa or inflammatory cells may contribute to the 18S signal. We could not identify better internal standards eliminating this problem. Nonetheless, the RT-PCR data of total liver support the results obtained by immunohistochemistry.

In a previous report on expression of hepatobiliary transporters in human cholestatic liver diseases, no up-regulation of MDR1, or MRP3 was seen.\textsuperscript{19} We show in the present study a 5-fold up-regulation of MDR1 and a 10-fold up-regulation of MRP3 in liver specimens of PBC patients. This difference is probably related to the stage of the disease: most of our specimens were in a late stage, since they were mainly pre-transplant hepatectomy specimens. In addition, the dilution of the antibodies until the critical staining level permitted us to evaluate a slight up- or down-regulation. Zollner \textit{et al.}\textsuperscript{19} used fluorescence staining methods and only one dilution, which does not permit this evaluation.

We have also studied the ABC transporter gene expression in livers of rats treated with 2-acetylaminofluorene (2-AAF) followed by 70% partial hepatectomy. This activates the progenitor cell compartment. These cells expressed high levels of Mrp1 and Mrp3 (Ros \textit{et al.}, submitted). This correlates with the high expression levels of MRP1 and MRP3
observed in this study. Unlike the human samples, rat liver progenitor cells in the 2-AAF/PHx model did not express high levels of P-glycoproteins. This difference may be related to the severity of damage in the human liver specimens compared to the rat model. In addition, MDR1 expression levels in human liver are in general higher than Mdr1a/b levels in laboratory animals.

In conclusion, using immunohistochemistry and dilution series, we showed a strong up-regulation of apical MDR1 and basolateral MRP1 and MRP3 in human hepatocytes and in progenitor cell-related bile ductules, during hepatitis or chronic cholestasis. We hypothesize that this reflects a protective mechanism against the accumulation of toxic bile constituents and may render these cells resistant to oxidative stress.

4.6 Acknowledgment

This study was supported by a grant of the Netherlands Organization for Scientific Research (NWO 902-23-191) and by a grant of the “Fonds voor Wetenschappelijk Onderzoek” Flanders.
MDR1, MRP1, and MRP3 expression in human hepatic progenitor cells

4.7 References


