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Animal Model

Mice with Homozygous Disruption of the *mdr2* P-Glycoprotein Gene
A Novel Animal Model for Studies of Nonsuppurative Inflammatory Cholangitis and Hepatocarcinogenesis

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The mouse* mdr2 *gene (and its human homologue* MDR3, *also called* MDR2*) encodes a P-glycoprotein that is present in high concentration in the bile canalicular membrane of hepatocytes. The 129/OlaHsd mice with a homozygous disruption of the* mdr2 *gene (−/− mice) lack this P-glycoprotein in the canalicular membrane. These mice are unable to secrete phospholipids into bile, showing an essential role for the mdr2 P-glycoprotein in the transport of phosphatidylcholine across the canalicular membrane. The complete absence of phospholipids from bile leads to a hepatic disease, which becomes manifest shortly after birth and shows progression to an end stage in the course of 3 months. The liver pathology is that of a nonsuppurative inflammatory cholangitis with portal inflammation and ductular proliferation, consistent with toxic injury of the biliary system from bile salts unaccompanied by phospholipids. Thus, the* mdr2 *(−/−) mice can serve as an animal model for studying mechanisms and potential interventions in nonsuppurative inflammatory cholangitis (in a generic sense) in human disease, be it congenital or acquired. When the mice are 4 to 6 months of age, preneoplastic lesions develop in the liver, progressing to metastatic liver cancer in the terminal phase. The* mdr2 *(−/−) mice therefore also provide a tumor progression model of value for the study of hepatic carcinogenesis. Interestingly, also in this regard, the model mimicks human disease, because chronic inflammation of the biliary system in humans may similarly carry increased cancer risk. (Am J Pathol 1994, 145:1237–1245)

MDR genes belong to a small family of closely related genes, with two members in humans[1–3] and three members in rodents,[4–7] that encode for cell membrane proteins known as P-glycoproteins (P-gps). The human *MDR1* gene[2,3] and the mouse *mdr1* (or *mdr1b*) and *mdr3* (or *mdr1a*) genes[4,6] encode P-gps that mediate the increased efflux of intracellular hy-

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drophobic drugs observed in multidrug resistance (MDR).[8] The human *MDR3* (also called *MDR2*)[1] gene product and its mouse homologue the mdr2 P-gp, however, do not confer MDR to cells and until recently their transport function was poorly understood.

Its typical distribution pattern in adult liver, with an exclusive expression at the canalicular membrane of hepatocytes,[9–11] suggested a role for the mdr2 P-gp in biliary excretion. Proof for such a role was recently obtained when homozygous mice, in which the *mdr2* gene had been disrupted, were generated:[12] phospholipid excretion in bile was undetectable in the homozygous (–/–) *mdr2* mice and reduced to 60% of normal in the heterozygous (+/–) *mdr2* mice. Other abnormalities in the bile included a decrease of the biliary glutathione (down to 14% of controls) and cholesterol secretion (down to 7%) in the *mdr2* (–/–) mice, whereas in the *mdr2* heterozygotes these values were comparable to the wild-type animals. The bile acid and conjugated bilirubin output in the *mdr2* (–/–) mice was not different from the *mdr2* (+/–) and (+/+) mice, but the bile flow in *mdr2* (–/–) mice was twice that of the heterozygous *mdr2* and normal wild-type mice, presumably due to ductular proliferation (see below). In the *mdr2* (–/–) mice, serum bilirubin was variably (3- to 20-fold) elevated, approximately half of it being conjugated. This can be attributed to leakage from damaged hepatocytes or bile canaliculi. Liver function tests were also moderately (3- to 6-fold) elevated, consistent with damage of the liver parenchyma. In the *mdr2* (+/–) mice, serology was comparable to the wild-type mice and within normal limits. All available results indicate that the primary defect in the *mdr2* (–/–) mice is their inability to secrete phospholipids into bile.

Apparently, the mdr2 P-gp is essential for this process and this protein may act as a phospholipid translocator or flippase,[13] although a more indirect effect of the protein on phospholipid transport is not excluded.[12] The secretion of hydrophobic bile salts in the absence of phospholipid results in damage of membranes[14,15] and liver disease.[12] This article gives a detailed description of the accompanying liver pathology and considers potential applications of the *mdr2* (–/–) mouse as an animal model of human hepatobiliary disease.

Materials and Methods

Animals

The generation of homozygous *mdr2* (–/–) mice lacking a functional *mdr2* gene has been described in detail previously.[12] In this study, mice homozygous (–/–) and heterozygous (+/–) for the disruption of the *mdr2* gene and normal wild-type (+/+) mice (129/OlaHsd; Harlan UK Limited, Bicester, UK) were compared. The study group was comprised of 66 mice with ages ranging from 2 days to 1.5 years. As a routine procedure, the genotype of the mice was checked by Southern blot analysis of genomic DNA isolated from individually collected tails at sacrifice. DNA isolation and Southern blot analysis were performed according to standard techniques.[16,17] The mice were fed a standard diet (AM-II diet; Hope Farms, The Netherlands) and water ad libitum. Animal welfare was in accordance with the institutional guidelines of the University of Amsterdam.

Histochemistry

Tissue samples were fixed by immersion in a mixture of ethanol, acetic acid, and formalin (8:1:2, v/v).[18] Five-micron thick serial paraffin sections were stained according to standard histochemical procedures with hematoxylin and eosin (H&E), periodic acid Schiff (PAS), periodic acid Schiff after diastase digestion (PAS-D), Gomorri's reticulin stain, Perl's iron, and van Giesson's elastic staining. ATPase enzyme histochemistry was conducted on selected cases with large nodules in the liver using 5-μ thick cryostat sections.

Immunohistochemistry

Immunoperoxidase staining was performed with a panel of polyclonal (Pabs) and monoclonal antibodies (MAbs) raised against different antigens (Table 1). Tissue processing and immunohistochemistry were performed according to standard procedures (for details see Table 1). Briefly, endogenous peroxidase activity was blocked by incubation of sections in 0.3% (v/v) H_2O_2 in methanol for 20 minutes. Nonspecific binding of immunoglobulins (Igs) was blocked by preincubation with normal goat serum (Dako A/S, Copenhagen, Denmark). Before immunostaining with the antikeratin Pab, sections were predigested with 0.25% (w/v) pepsin (Sigma, St. Louis, MO) in 10 mmol/L HCl for 15 minutes at 37 C. Likewise, sections were pretreated with 10 mmol/L citrate buffer (pH 6) for 2 × 5 minutes at 100 C when the anti-Ki-67 Pab was used. Antibody binding on sections was visualized using 0.1% (w/v) 3,3′-diaminobenzidine tetrahydrochloride (Sigma) dissolved in 50 mmol/L Tris-HCl (pH 7.6) containing 0.03% (v/v) H_2O_2 as the chromogen. Color was developed for 7 minutes. In most experiments sections were counterstained with hematoxylin.

Table 1. *Primary Pabs and MAbs Used in this Study*

Antibody	Supplier	Specificity	References*
P-gps			
Pab REG3		MDR3 and mdr2 P-gp	Smit et al[11,12]
MAb C219		All mouse P-gps	Georges et al[19]
Proliferation markers			
Pab Ki-67	Dako A/S, Denmark (*A 047*)	395- and 345-kd polypeptides of a cell proliferation-associated nuclear antigen	Key et al[20] Schlüter et al[21]
Intermediate filament proteins			
Pab keratin	Dako A/S, Denmark (*Z 622*)	Keratin subunits of 58, 56, and 52 kd and less abundantly subunits of 60, 51, and 48 kd	Steiner[22] Franke et al[23]
Serum proteins			
Pab α_1-fetoprotein	Dako A/S, Denmark (*A 008*)	α_1-Fetoprotein	Jacobsen et al[24]
Pab α_1-fetoprotein	Obtained from Dr. S.H. Yap	α_1-Fetoprotein	Selten et al[25]
Metabolic markers			
Pab glutamine synthose		Glutamine synthase	de Groot et al[26]

* References refer to the fixation and immunohistochemical staining method applied to reveal the expression of the antigen.

Commercially available unconjugated secondary antibodies and peroxidase antiperoxidase immunocomplexes were purchased from Nordic (Nordic, Tilburg, The Netherlands). Biotinylated swine to rabbit Igs and horseradish peroxidase-conjugated streptavidin were obtained from Dako.

Electron Microscopy

The livers of 20 *mdr2* (−/−) and of 13 control (+/+) mice were prepared for electron microscopy. They covered the same age range, ie, from 2 days to 1.5 years, as the animals studied light microscopically. Routinely, whole livers were perfused with the aid of a peristaltic pump via the portal vein with 200 mmol/L cacodylate buffer, followed after 30 seconds by 2% (w/v) paraformaldehyde in the same buffer. In a few cases, a single lobe of a liver intended for light microscopic examination was perfused by hand via the portal branch. Dissected tissue blocks were postfixed with 1% OsO_4 and embedded in LX-112 resin. Before uranyl and lead staining, thin sections were briefly treated with diluted tannic acid to increase the contrast of both glycogen and extracellular elements.[27] In a number of samples peroxisomes were identified by means of ultrastructural catalase histochemistry.[28]

Light Microscopy

Various histopathological parameters (Figure 1B) were systematically assessed to evaluate the morphology of the liver pathology that develops in the *mdr2* (−/−) mice. Some of these parameters were determined by computerized image analysis using a light microscope equipped with a charge-coupled device monochrome video camera (AIS MX5). For the data analysis a standard image processing program (TEA Image Manager, DIFA Measuring Systems, The Netherlands)[29] was used.

Periportal and pericentral regions of the liver lobule were compared and arbitrarily defined as concentric layers of five hepatocytes thick surrounding the terminal branches of the portal vein or the branches of the hepatic vein.

Results and Discussion

Nonsuppurative Inflammatory Cholangitis in the mdr2 *(−/−) Mice and Potential Human Homologues*

The *mdr2* (−/−) mice are indistinguishable from the heterozygous *mdr2* (+/−) and wild-type (+/+) mice in external appearance, mortality, growth rate, and weight during the first year of age.[12] The fertility of the females appears to decrease at ages more than 4 months. Thus far organs other than the liver have not shown any abnormality. All tissues were examined by light microscopy at various times after birth and compared with those of heterozygous and wild-type mice. Special attention was paid to organs where *mdr2* expression is predominantly found (ie, spleen, heart, muscle, and adrenal).[12] Sex differences were not seen. No morphological, difference between the *mdr2* (+/−) and the wild-type control mice was observed.

The liver of the *mdr2* (−/−) mice looks anatomically normal at birth. In accordance with the literature,[30] the bile canaliculi of all neonatal mice (−/−, +/−, and

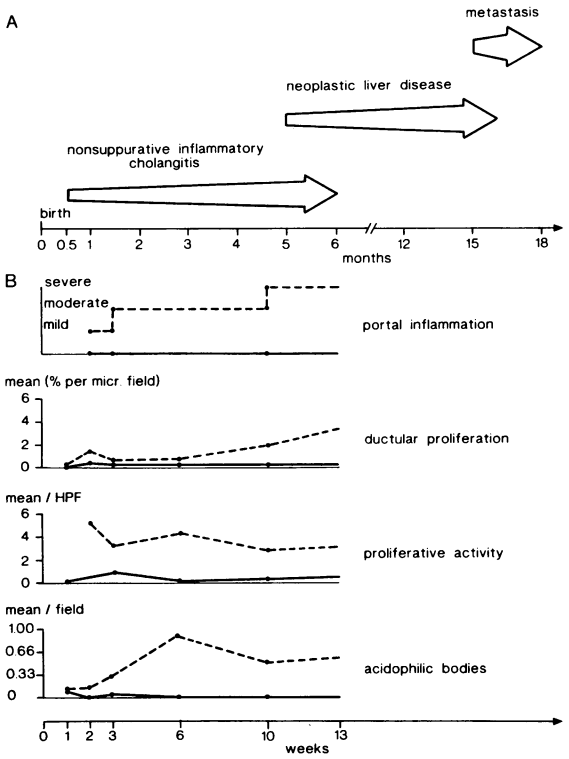

Figure 1. A: *Schematic diagram of the nature and time sequence of the liver disease in the mdr2 P-gp-deficient mice.* B: *Evolution of the main histological parameters characterizing the development of nonsuppurative inflammatory cholangitis during the first 3 months. The degree of portal inflammation was graded as absent (no inflammatory cells present), light (only scattered inflammatory cells present in the mesenchyme of the portal triads), moderate (increasing inflammatory infiltrate in the portal triads associated with cholangiolitis, ie, inflammatory cells invading bile ducts, no fibrosis present), and severe (in addition to the inflammatory infiltrate, fibrosis is also present). As a measure for the extent of ductular proliferation, the size of the ductular compartment (delineated by a positive staining for the antikeratin Pab; see Figure 3B) was assessed in 10 microscopic fields at ×250 magnification using computerized image analysis. The proliferative activity was evaluated by the Ki-67 labeling index (ie, the number of positively stained nuclei for the anti-Ki-67 Pab per high-power field [HPF]), and the number of acidophilic ('Councilman') bodies were quantified in 20 randomly chosen microscopic fields at ×400 magnification. Dotted lines,* mdr2 (-/-) *mice; straight lines, wild-type control* (+/+) *mice.*

+/+) are wide and relatively smooth. In (+/+) and (+/−) mice these immature bile canaliculi develop within 2 to 3 weeks into the adult type, ie, their diameters decrease with a concomitant increase in the number of microvilli. In contrast, the immature phenotype consistently persists in the adult *mdr2* (−/−) mice and the canaliculi remain wide and smooth, whereas an excessive number of canalicular cross-sections indicates tortuosity (Figure 2). In addition, in the pericanalicular ectoplasm deposition of microfilamentous material is often present. These electron microscopic findings resemble those of cholestasis and are in itself nonspecific. Presumably, it is therefore best considered a secondary phenomenon due to the increased flow of toxic bile salts.

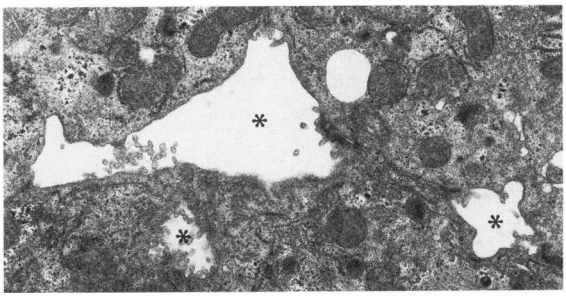

Figure 2. *Electron microscopy of the liver of a 1-year-old mdr2 P-gp-deficient mouse. The presence of three bile canalicular profiles (asterisks) in one field indicates an increased tortuosity of bile canaliculi. Note that the central bile canalicular profile is excessively wide and that there are only relatively few microvilli. Original magnification ×9,200.*

When these ultrastructural differences between the *mdr2* (−/−) and (+/+) mice become clear, light microscopic alterations also begin to arise, and at 2 to 3 weeks light microscopy will distinguish the *mdr2* (−/−) mice from the controls ((+/−) and (+/+)). There is ductular proliferation and portal inflammation with a mixed inflammatory infiltrate; the bile duct epithelium shows reactive changes and presence of intraepithelial inflammatory cells (Figure 3). Throughout the lobule an increased number of acidophilic bodies and mitotic figures is seen. The proliferative activity assessed by Ki-67 labeling is increased both in the pericentral and periportal area. Because the first visible morphological changes concern the canaliculi and bile ductuli, presumably this part of the biliary tract is affected first. The light microscopic changes progress ultimately to a relatively stable end stage in the course of approximately 3 months. Although there is considerable expansion of the portal triad, biliary cirrhosis with porto-portal fibrous septa is never encountered.

Immunohistochemistry with the anti-REG3 antibody specific for mdr2 P-gp[11] shows no canalicular staining in *mdr2* (−/−) mice, whereas the positive immunostaining with the C219 antibody that recognizes all three mouse P-gps, remains.[11] This confirms the specific loss of the canalicular mdr2 P-gp, which leads to the complete loss of biliary phospholipid excretion.[12] The liver pathology is that of a nonsuppurative inflammatory cholangitis in a generic sense[31] and it is consistent with the postulated mechanism of toxic injury by bile salts due to a lack of biliary phospholipids.[12,32] That explains why morphological change does not arise until some time after birth when the bile salt secretion starts to build up.[33]

Nonsuppurative inflammatory duct lesions form the morphological hallmark of an important category of pathological conditions in humans, both in congenital and acquired hepatobiliary diseases.[31,34] Several of

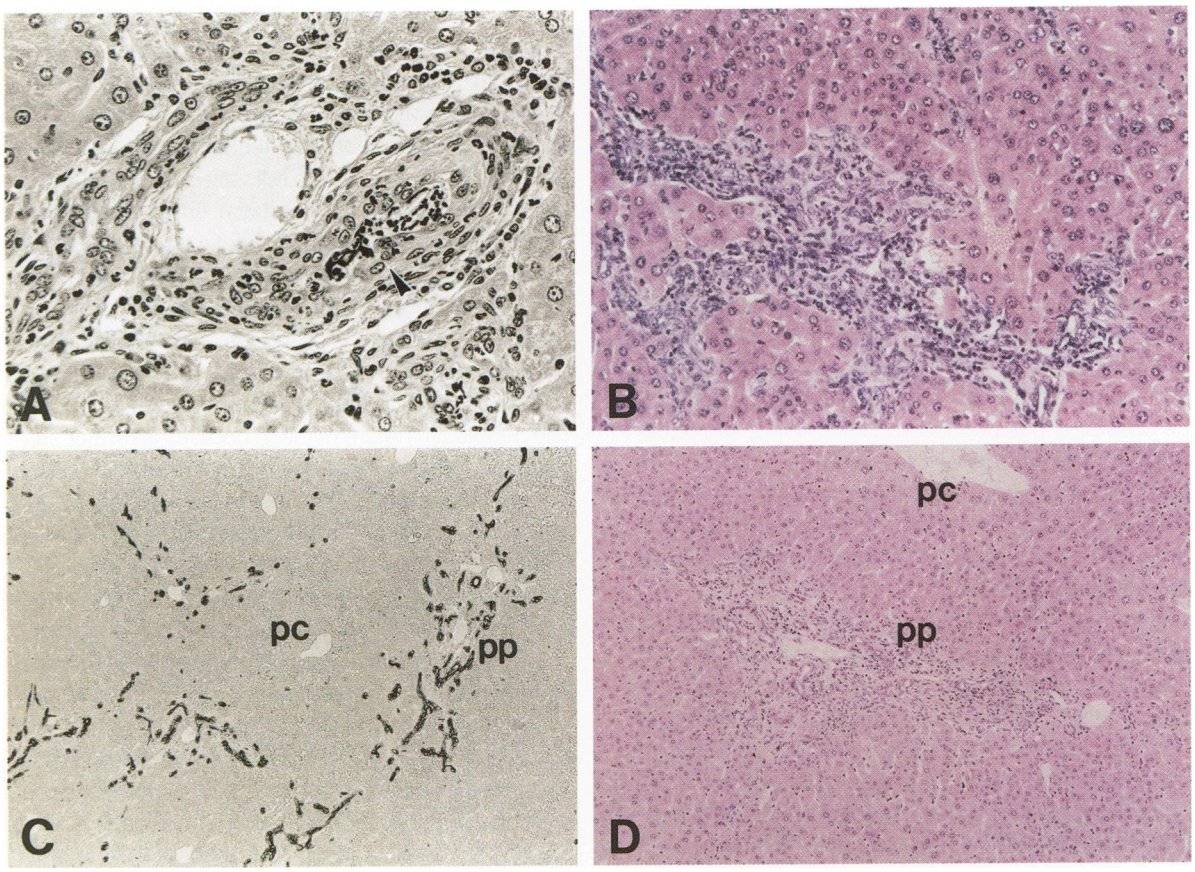

Figure 3. *Microscopy of the liver of a 3-months-old mdr2 P-gp-deficient mouse.* A: *Portal inflammation with inflammatory cells invading bile ducts (arrowhead) and fibrosis; H&E staining.* C: *Ductular proliferation of bile ducts extending into the parenchyma of the periportal region. Immunoperoxidase staining with the antikeratin Pab.* B, D: *Light microscopic overview of the cholangitis accompanied by prominent portal expansion due to inflammation, ductular proliferation, and fibrosis; H&E staining. pp, Periportal region; pc, pericentral region. Original magnification:* A, ×*100;* B, ×*40;* C *and* D, ×*25.*

these conditions are poorly understood and it is conceivable that a primary defect in biliary phospholipid excretion as the underlying mechanism has been overlooked.[31,34–36] A genetic defect, but also autoimmunity or other insults (eg, infection and drugs), might interfere with phospholipid excretion. The human MDR3 P-gp and the murine mdr2 P-gp are very similar in all essential properties. Their amino acid sequences are more than 90% identical and their tissue distribution is the same.[11] The MDR3 P-gp is also abundantly present in the hepatocyte canalicular membrane,[11] and it is therefore very likely that it is essential for phospholipid secretion into bile in humans too. A complete absence of the MDR3 P-gp could be expected to result in rapidly progressive disease, incompatible with longstanding survival; partial defects might lead to a milder picture that could be provoked by factors that cause impairment of P-gp activity or increase of bile salt flow. Importantly, human bile salts are more hydrophobic than those of rodents and might, therefore, lead to more aggressive disease and a more destructive or obliterative type of ductular lesions.

Of the neonatal conditions, progressive familial intrahepatic cholestasis ('Byler'-like pictures),[37–39] biliary atresia, and paucity of bile ducts need particular consideration.[31,34,40] It is at least remarkable that obliteration or destruction of the intrahepatic ducts apparently only takes place some time after birth. These ducts become patent during the first weeks of life and to our knowledge obliteration at autopsy in a stillborn has never been described.[40]

Acquired destructive cholangiopathies in adults, in which the pathology shows resemblance with the above category of neonatal diseases, are pictures that resemble primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC) but also unexplained vanishing bile duct syndromes.[31,34–36,41,42] These conditions too are mostly not well understood and, therefore, need consideration. In typical PBC

and PSC, we and others have not observed a decreased biliary phospholipid excretion.[43] Finally, interference with MDR3 P-gp function or other forms of inhibition of biliary phospholipid secretion could also underlie some of the drug reactions leading to nonsuppurative cholangitis.[31,44] Whether loss of MDR3 function plays a role in any of the above clinical disorders characterized by cholangiocellular destruction, remains of course a theoretical and speculative concept until proven otherwise. Obviously, any other process that interferes with phospholipid synthesis or transport of phospholipid into bile could give rise to a phenocopy of MDR3 P-gp deficiency. For instance, the possibility has been raised that the hepatic phosphatidylcholine transfer protein is required for the transport of newly synthesized phosphatidylcholine to the canalicular membrane.[12,45] A defect in this protein might therefore also interfere with the secretion of phospholipid into bile.

Even if in humans the very same defect as in the *mdr2* (–/–) mouse is not easily traceable, the induced inflammatory cholangitis in the mouse should serve as an animal model to study potential interventions in the complex disease category of nonsuppurative cholangitis. Modulation of bile salt composition in favor of hydrophilic bile salts unable to elute phospholipid from membranes might diminish cellular injury, a therapeutic effect that might explain why some patients with PBC, PSC, and analogous diseases benefit from ursodeoxycholate administration.[46–48]

Hepatocarcinogenesis in the mdr2 *(–/–) Mice*

At the age of approximately 4 to 6 months the *mdr2* (–/–) mice start to develop multiple foci in the liver parenchyma, culminating in grossly visible nodular outgrowths in the hepatectomy specimens (Figure 4, compare A with B). The irregularly enlarged livers show multiple tumors, often with necrosis and hemorrhage. Using light microscopy, the lesions consist of sheets of atypical hepatocytes with loss of the preexisting architecture and reticulin pattern. There is strong cytonuclear polymorphism, an increased number of mitotic figures, and apoptotic bodies (Figure 5, B and C). At this stage the lesions appear relatively circumscribed and vasoinvasion has not been observed with certainty but the lesions do fulfill microscopic criteria used in chemically induced hepatocarcinogenesis to diagnose neoplastic nodules.[49,50] The neoplastic nature of these nodules is further supported by the observed decrease of PAS-positive glycogen in these lesions, the loss of the ATPase staining reaction in enzyme histochemistry, and the positive glutamine synthase immunostaining (Figure 5A);[50,51] in one case subcutaneous transplantation of preneoplastic liver tissue resulted in a tumorous outgrowth.

The nodules show a highly variable ultrastructural picture. Some are made up of small cells without peroxisomes but others consist of cells that can be recognized as hepatocytes with numerous peroxisomes, as verified by catalase histochemistry. Also in the preexisting liver cells of older *mdr2* (–/–) mice, the number of peroxisomes is often increased. This is of interest, because it has been postulated that proliferation of peroxisomes serves as an endogenous initiator of neoplastic transformation in the liver through the production of oxygen radicals.[52–54] Whether peroxisome proliferation causes cancer or is part of the genetic reprogramming caused by cancer remains controversial, however.[53] Replication of DNA is not a flawless process and increased cell turnover increases the rate of somatic mutation. It is therefore possible that the hepatocarcinogenesis in the *mdr2*

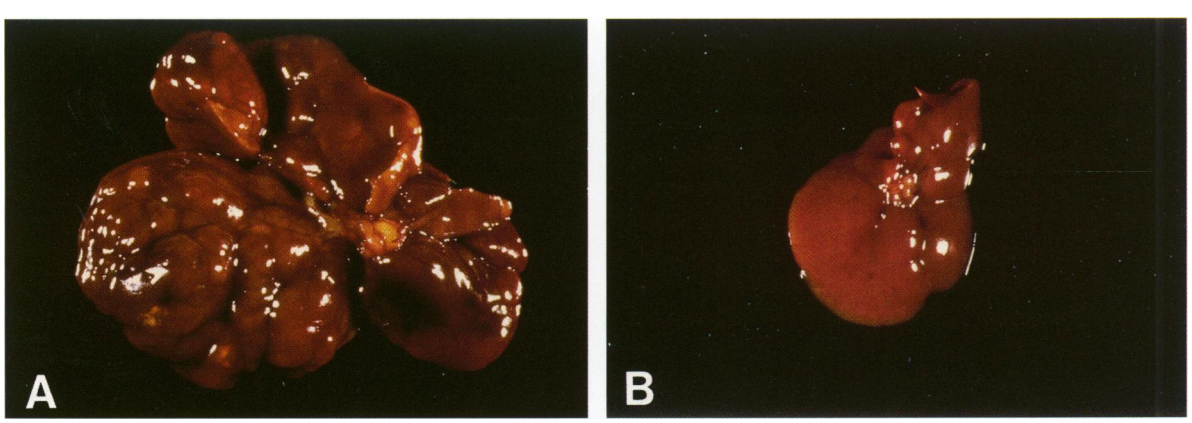

Figure 4. *Macroscopy of the liver of a 6-months-old mdr2 P-gp-deficient mouse* (**A**) *compared with a wild-type control mouse* (**B**). *Note the nodularity of the* (7- *to 8-fold*) *enlarged liver in the* mdr2 (–/–) *mouse.*

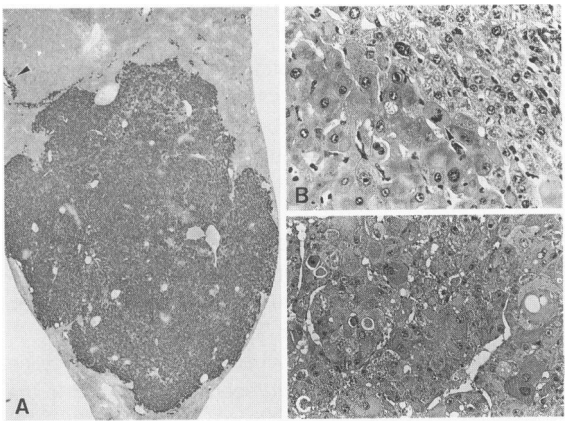

Figure 5. *Microscopy of the liver of a 1-year-old mdr2 P-gp-deficient mouse showing the features of a neoplastic nodule.* **A:** *Positive immunoreactivity for glutamine synthase. The original pericentral localization of glutamine synthase positivity in the preexistent liver parenchyma is indicated by the arrowhead. Immunoperoxidase staining with the antiglutamine synthase Pab.* **B:** *Cytonuclear polymorphism and mitotic figures (arrowhead) of hepatocytes. The lesion is relatively well demarcated; H&E staining.* **C:** *In addition, there is loss of the original trabecular architecture with a tendency of the liver cells to grow in sheets; H&E staining. Original magnification:* **A**, ×*6.25;* **B**, ×*100;* **C**, ×*25.*

(−/−) mouse only reflects the chronic liver damage and the ensuing increased rate of cell turnover. Hepatocarcinogenesis has also been seen in other conditions resulting in widespread liver necrosis in rodents, eg, choline-deficient diet.[54] So far, the two oldest (1.5 years of age) *mdr2* (−/−) mice showed lung metastasis and the presence of many catalase-positive peroxisomes in these secondary tumors unequivocally confirmed their hepatocyte derivation (Figure 6). Thus, the natural history of the liver disease that accompanies the homozygous disruption of the murine *mdr2* allele apparently constitutes a tumor progression model of spontaneous hepatocarcinogenesis. Also, in this regard, a link with human disease can be seen because chronic inflammation of the biliary system in humans may similarly carry cancer risk, although in that event the cholangiocellular phenotype appears as the preferred lineage.

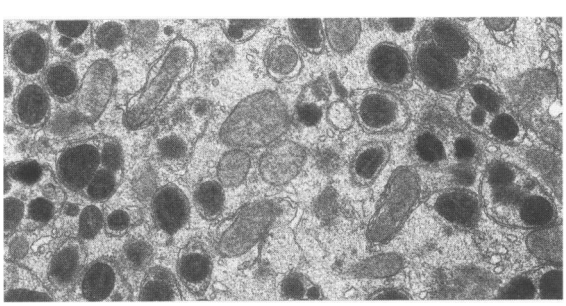

Figure 6. *Electron microscopy of lung metastasis of a 1.5-year-old mdr2 P-gp-deficient mouse. Only a small cytoplasmic part of one cell is shown. Note that peroxisomes are abundantly present. Catalase histochemistry has been used to increase the electron density of peroxisomes. Original magnification* ×*21,000.*

Further studies are needed to delineate the precise nature of the neoplasms in the livers of the *mdr2* (−/−) mice but the mouse could serve as a valuable adjunct in the current debates on liver stem cells.[55–57] Importantly, α_1-feloprotein positivity was not seen in the *mdr2* (−/−) mice; at the terminal ends of the bile ductules, epithelial cells were, however, found that bordered directly on the hepatocytes, often in irregular groups without a recognizable lumen. These cells might therefore be transition duct cells.[58] Using recently described ultrastructural criteria,[59] we could not easily detect oval cells by electron microscopy. The glutamine synthase positivity that accompanies the neoplastic transformation may also point to a specific cell lineage but then from a pericentral origin.[51,60]

In conclusion, the *mdr2* (−/−) mouse not only serves as an interesting model for studies of nonsuppurative inflammatory cholangitis, but further studies of the tumors that eventually arise in the livers of these mice may also contribute to a better understanding of hepatocarcinogenesis.

Availability and Biological Features

The mdr2 P-gp-deficient mouse is available for other investigators. The 129/OlaHsd strain is generally regarded as a poor breeder and the mice generate limited offspring. The fertility in the female *mdr2* (−/−) mice even decreases after 4 months. Therefore, the 129/OlaHsd *mdr2* (−/−) mice were intercrossed and further backcrossed to FVB/NOlaHsd mice. The FVB/N *mdr2* (−/−) mice have a phenotype identical to that of the 129/OlaHsd during the first 3 to 4 months. The long-term picture, however, is yet unknown.

Acknowledgments

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