Multidrug Resistance–Associated Proteins Are Crucial for the Viability of Activated Rat Hepatic Stellate Cells

Rebekka A. Hannivoort,[1]* Sandra Dunning,[1]* Sara Vander Borght,[2] Ben Schroyen,[3] Jannes Woudenberg,[1] Fiona Oakley,[4] Manon Buist-Homan,[1] Fiona A. J. van den Heuvel,[1] Mariska Geuken,[1] Albert Geerts,[3] Tania Roskams,[2] Klaas Nico Faber,[1] and Han Moshage[1]

Hepatic stellate cells (HSCs) survive and proliferate in the chronically injured liver. ATP-binding cassette (ABC) transporters play a crucial role in cell viability by transporting toxic metabolites or xenobiotics out of the cell. ABC transporter expression in HSCs and its relevance to cell viability and/or activation have not been reported so far. The aim of this study was to investigate the expression, regulation, and function of multidrug resistance–associated protein (Mrp)-type and multidrug resistance protein (Mdr)–type ABC transporters in activated rat HSCs. Rat HSCs were exposed to cytokines or oxidative stress. ABC transporter expression was determined by quantitative polymerase chain reaction and immunohistochemistry. HSCs were exposed to the Mdr inhibitors verapamil and PSC-833 and the Mrp inhibitor MK571. Mdr and Mrp transporter function was evaluated with flow cytometry. Apoptosis was determined by activated caspase-3 and acridine orange staining, and necrosis was determined by Sytox green nuclear staining. An *in vivo* model of carbon tetrachloride (CCl_4)–induced liver fibrosis was used. With respect to hepatocytes, activated HSCs expressed high levels of Mrp1 and comparable levels of Mrp3, Mrp4, Mdr1a, and Mdr1b but not the hepatocyte-specific transporters bile salt export pump, Mrp2, and Mrp6. Mrp1 protein staining correlated with desmin staining in livers from CCl_4-treated rats. Mrp1 expression increased upon activation of HSCs. Cytokines induced Mdr1b expression only. Oxidative stress was not a major regulator of Mdr and Mrp transporter expression. Activated HSCs became necrotic when exposed to the Mrp inhibitors. *Conclusion:* Activated HSCs contain relatively high levels of Mrp1. Mrp-type transporters are required for the viability of activated HSCs. Mrp-dependent export of endogenous metabolites is important for the survival of activated HSCs in chronic liver diseases. (HEPATOLOGY **2008;48:624-634.**)

Abbreviations: α-SMA, α-smooth muscle actin; ABC, ATP-binding cassette; Ad5IκBAA, adenovirus encoding for dominant-negative inhibitor of nuclear factor-κB-α; Ad5LacZ, adenovirus encoding for β -galactosidase; Bsep, bile salt export pump; CCl_4, carbon tetrachloride; CM, cytokine mixture; CMFDA, 5-chloromethylfluorescein diacetate; EMSA, electrophoretic mobility shift assay; GFAP, glial fibrillary acidic protein; H_2O_2, hydrogen peroxide; HO-1, heme-oxygenase-1; HPC, hepatic progenitor cell; HSC, hepatic stellate cell; IκB, inhibitor of nuclear factor-κB; iNOS, inducible nitric oxide synthase; Mdr, multidrug resistance protein; Mrp, multidrug resistance–associated protein; mRNA, messenger RNA; NF-κB, nuclear factor-κB; PBS, phosphate-buffered saline; PCR, polymerase chain reaction.

From the [1]Department of Gastroenterology and Hepatology, University Medical Center Groningen, University of Groningen, Groningen, the Netherlands; [2]Department of Morphology and Molecular Pathology, University of Leuven, Leuven, Belgium; [3]Laboratory for Cell Biology, Free University Brussels, Brussels, Belgium; and [4]Liver Group, Institute of Cellular Medicine, Newcastle University, Newcastle upon Tyne, United Kingdom.

This research was partially funded by the Dutch Digestive Foundation and the J. K. de Cock Stichting.

Some of the results were presented at the 2004 Annual Meeting of the American Association for the Study of Liver Diseases in Boston, the 2005 Annual Meeting of the American Association for the Study of Liver Diseases in San Francisco, and the 2006 American Association for the Study of Liver Diseases Single Topic Conference on Hepatic Fibrosis in Virginia.

**These authors contributed equally to this work.*

Received June 28, 2007; accepted March 25, 2008.

Address reprint requests to: Rebekka A. Hannivoort, Department of Gastroenterology and Hepatology, University Medical Center Groningen, University of Groningen, P.O. Box 30.001, 9700 RB Groningen, the Netherlands. E-mail: r.a.hannivoort@med.umcg.nl; fax. +31-50-3619306.

Published online in Wiley InterScience (www.interscience.wiley.com).

DOI 10.1002/hep.22346

Potential conflict of interest: Nothing to report.

During chronic liver injury, liver cells are exposed to bile acids, oxidative stress, and cytokines.[1,2] Under these conditions, hepatic stellate cells (HSCs) and hepatic progenitor cells (HPCs) proliferate,[3-5] whereas hepatocytes die. Hepatocyte death leads to impaired liver function, whereas HSC activation and proliferation lead to the development of liver fibrosis and cirrhosis.[6] In recent years, much progress has been made in elucidating the mechanisms involved in activation and proliferation of HSCs and the mechanisms of hepatocyte death induced by bile acids, oxidative stress, and cytokines.[7-9] Also, recent studies have provided evidence that liver fibrosis and even cirrhosis can be reversed by the induction of HSC apoptosis.[10] However, virtually nothing is known about the protection mechanisms of HSCs against the toxic agents that are present in chronic liver injury.

ATP-binding cassette (ABC) transporters play a major role in the multidrug-resistant and proliferative phenotype of many cancer cells. ABC transporters also play an extremely important role in liver physiology. ABC transporters in the liver are involved in hepatobiliary transport of metabolites of endogenous and exogenous compounds, phospholipids, cholesterol and bile acids in hepatocytes, bile duct epithelial cells, and enterocytes.[11] Two subclasses of these ABC transporters, multidrug resistance protein (Mdr)–type transporters (ABCB subclass) and multidrug resistance–associated protein (Mrp)–type transporters (ABCC subclass), protect the proliferating cell from xenobiotics, including chemotherapeutics, and endogenous cell stress by transporting toxic metabolites out of the cell.[12] Mdr-type transporters are capable of removing many hydrophobic substrates from the cell, whereas Mrp-type transporters, particularly Mrp1, transport organic anions such as glutathione, glucuronate, and sulfate conjugates.[13] Recently, high expression of several ABC transporters in HPCs has been described in various liver pathologies. Most prominent is the expression of Mdr1b, Mrp1, Mrp3, and breast cancer resistance protein in HPCs.[14,15] It is assumed that the high expression of these transporters enables HPCs to survive liver injury and to proliferate because these ABC transporters export endogenous and exogenous toxic compounds out of the cells. Like HPCs, HSCs survive and proliferate in the chronically injured liver. However, nothing is known about the role of ABC transporters in HSCs. Therefore, the aim of the present study was to investigate the expression, regulation, and function of ABC transporters in activated HSCs. Our study demonstrates that ABC transporters are expressed in HSCs and play an important role in the protection against cell death.

Materials and Methods

Animals. Specified pathogen-free male Wistar rats were purchased from Harlan (Zeist, the Netherlands). They were housed under standard laboratory conditions with free access to standard laboratory chow and water. Each experiment was performed following the guidelines of the local committee for care and use of laboratory animals.

Liver fibrosis was induced in male Wistar rats with a body weight of 250-300 g by the administration of carbon tetrachloride (CCl_4) for 2 weeks as described previously.[16] Liver specimens were snap-frozen in liquid nitrogen–cooled isopentane and stored at −70°C until use.

Rat HSC Isolation and Culture. HSCs were isolated from male Wistar rats (500-600 g) by pronase and collagenase perfusion of the liver followed by Nycodenz gradient (12% wt/vol) centrifugation as described previously.[17]

Cells were then cultured in Iscove's modified Dulbecco's medium with Glutamax (Invitrogen, Breda, the Netherlands) supplemented with 20% heat-inactivated fetal calf serum (Invitrogen), sodium pyruvate (Invitrogen), nonessential amino acids (Invitrogen), 50 μg/mL gentamicin (Invitrogen), and 250 U/mL Nystatin (Sanofi-Synthelabo, Maassluis, the Netherlands) in a humidified atmosphere containing 5% CO_2 at 37°C. Primary HSC cultures were allowed to grow to confluence, subcultured by trypsinization, and then cultured in Iscove's medium with supplements as described previously, but without Nystatin. Prior to experiments, HSC were serum-starved for 24 hours.

Human HSC Isolation and Culture. Human HSCs were extracted from the margins of normal human livers resected for colonic metastatic disease as previously described.[18] Human HSCs were used for experimentation after activation and before the fourth passage. The use of human liver tissue for scientific investigation was approved by the UK South and West Local Research Ethics Committee and was subject to patient consent.

Hepatocyte Isolation and Culture. Primary rat hepatocytes were isolated from male Wistar rats (200-250 g) with a 2-step collagenase perfusion as described previously.[19] Cell viability was consistently more than 90% as determined by trypan blue exclusion. Isolated hepatocytes were plated at a density of 125,000 cells/cm^2 in William's medium E (Invitrogen) supplemented with 50 μg/mL gentamycin (Invitrogen). During the attachment period (4 hours), 50 nmol/L dexamethasone (UMCG Pharmacy) and 5% fetal calf serum (Invitrogen) were added to the medium. Cells were cultured in a humidified incubator containing 5% CO_2 at 37°C.

Experimental Design. Culture-activated HSCs (passage 2-3) were used for all experiments, unless indicated otherwise. Primary rat hepatocytes were used 18 hours after seeding.

HSCs were exposed to a cytokine mixture (CM) containing 20 ng/mL recombinant mouse tumor necrosis factor α (R&D Systems, Abingdon, United Kingdom), 10 ng/mL recombinant human interleukin-1β (R&D Systems), and 10 ng/mL recombinant rat interferon-γ (R&D Systems) for 6 hours. Oxidative stress was induced by the exposure of the cells to the intracellular superoxide anion donor menadione (2-methyl-1,4-naphthoquinone; Sigma Aldrich) or hydrogen peroxide (Merck, Haarlem, the Netherlands) at the indicated concentrations for 6 hours. The leukotriene D4 receptor antagonist MK571 (Kordia Life Sciences, Leiden, the Netherlands) was used as an Mrp inhibitor.[20] Verapamil (Sigma Aldrich) and the cyclosporin A analogue PSC-833 (Sigma Aldrich) were used as Mdr inhibitors.[21,22]

Adenoviral Constructs. The adenoviral constructs used were described previously.[23] The inhibitor of nuclear factor-κB (IκB) super-repressor adenovirus Ad5IκBAA contains an IκB construct in which serines 35 and 36 have been replaced by alanines. This mutated IκB cannot be phosphorylated and binds nuclear factor-κB (NF-κB) irreversibly, preventing translocation of NF-κB to the nucleus. Ad5LacZ, an adenovirus expressing the *Escherichia coli* β-galactosidase gene, was used as a control virus throughout the experiments. Cells were infected with an adenovirus at the indicated multiplicity of infection 24 hours before the start of the experiments.

RNA Isolation, Reverse-Transcription Polymerase Chain Reaction (PCR), and Real-Time PCR. RNA was isolated with Tri Reagent (Sigma Aldrich) according to the manufacturer's instructions. RNA was quantified with a Ribogreen fluorescent assay (Invitrogen). Reverse-transcription and real-time PCR were performed as described previously.[24] 18S RNA was used as an internal control. Primers and probes used for real-time PCR are listed in Tables 1 and 2.

Double Fluorescence Immunostaining. Double staining was performed with a sequential fluorescent method on 20-μm-thick rat liver cryostat sections. The cryostat sections were dried overnight at room temperature, subsequently fixed in acetone for 10 minutes, and finally washed in phosphate-buffered saline (PBS) immediately before use. Then, the sections were incubated with a mixture of normal rabbit serum and normal swine serum diluted 1:5 in PBS for 30 minutes.

Sections were incubated with primary antibodies against the ABC transporters Mrp1, Mrp3, and C219 (pan-P-glycoproteins) and with primary antibodies against the HSC markers desmin and glial fibrillary acidic protein (GFAP) at room temperature for 45 minutes, and these were followed by tetramethylrhodamine isothiocyanate–labeled swine anti-rabbit antibodies (Dako, Glostrup, Denmark) and finally fluorescein isothiocyanate–labeled rabbit anti-mouse antibodies (Dako). The antibodies were diluted in PBS. All incubation steps were followed by a wash in 3 changes of PBS. Sections were mounted with a medium containing *para*-phenylene diamine. Controls consisted of the omission of either primary antibodies or one of the secondary antibodies and reversal of the order of incubation with antibodies. No aspecific labeling could be detected.

Table 1. Sequences of Rat Polymerase Chain Reaction Primers and Probes Used for Real-Time Detection Polymerase Chain Reaction Analysis

Gene		Primers
18S	Sense	5′-cgg cta cca cat cca agg a-3′
	Antisense	5′-cca att aca ggg cct cga aa-3′
	Probe	5′-cgc gca aat tac cca ctc ccg a-3′
Mrp1	Sense	5′-agg ctt ctt ggc aaa tcc aa-3′
	Antisense	5′-caa gca gta atc ccg gaa ctc t-3′
	Probe	5′-tgg ccc cat tca ggc cgt g-3′
Mrp2	Sense	5′-gac gac gat gat ggg ctg at-3′
	Antisense	5′-ctt ctc atg gcc aag gaa gct-3′
	Probe	5′-ccc acc atg gag gaa atc cct gag g-3′
Mrp3	Sense	5′-tcc cac ttc tcg gag aca gta act-3′
	Antisense	5′-ctt agc atc act gag gac ctt gaa-3′
	Probe	5′-cag tgt cat tcg ggc cta cgg cc-3′
Mrp4	Sense	5′-tca gtg ttg gac aga gac agt tag tg-3′
	Antisense	5′-ctt ctc ccg gat ttt ctg ttg tat-3′
	Probe	5′-tca gtt ctc gga tcc aca ttt gca gtt g-3′
Mrp5	Sense	5′-cgg cta acc gcg tat ttc ag-3′
	Antisense	5′-aac gct ttg acc cag gca ta-3′
	Probe	5′-tag ccg cca cag acg acc gtg tc-3′
Mrp6	Sense	5′-ctc tcc cat tgg ctt ctt tga g-3′
	Antisense	5′-gtc cac atc cac tat gtc cgt ct-3′
	Probe	5′-tcg gga acc tgc tga acc gtt ttt c-3′
Mdr1a	Sense	5′-gca ggt tgg ctg gac aga tt-3′
	Antisense	5′-gga gcg caa ttc cat gga ta-3′
	Probe	5′-ccg cca gag ttc cca gca gca tg-3′
Mdr1b	Sense	5′-aaa cat ggc acg taa cca aag tt-3′
	Antisense	5′-aaa atg tgg ccc tgt tta atg att-3′
	Probe	5′-cac tgt taa agg taa ttt cat caa gac gag aag cct tc-3′
iNOS	Sense	5′-gtg cta atg cgg aag gtc atg-3′
	Antisense	5′-cga ctt tcc tgt ctc agt agc aaa-3′
	Probe	5′-ccc gcg tca gag cca cag tcc t-3′
HO-1	Sense	5′-cac agg gtg aca gaa gag gct aa-3′
	Antisense	5′-ctg gtc ttt gtg ttc ctc tgt cag-3′
	Probe	5′-cag ctc ctc aaa cag ctc aat gtt gag c-3′
α-SMA	Sense	5′-gcc agt cgc cat cag gaa c-3′
	Antisense	5′-cac acc aga gct gtg ctg tct t-3′
	Probe	5′-ctt cac aca tag ctg gag cag ctt ctc ga-3′
Bsep	Sense	5′-cca agc tgc caa gga tgc ta-3′
	Antisense	5′-cct tct cca aca agg gtg tca-3′
	Probe	5′-cat tat ggc cct gcc gca gca-3′

α-SMA indicates α-smooth muscle actin; Bsep, bile salt export pump; HO-1, heme-oxygenase-1; iNOS, inducible nitric oxide synthase; Mdr, multidrug resistance protein; and Mrp, multidrug resistance–associated protein.

Table 2. Sequences of Human Polymerase Chain Reaction Primers and Probes Used for Real-Time Detection Polymerase Chain Reaction Analysis

Gene		Primers
18S	Sense	5′-cgg cta cca cat cca agg a-3′
	Antisense	5′-cca att aca ggg cct cga aa-3′
	Probe	5′-cgc gca aat tac cca ctc ccg a-3′
MRP1	Sense	5′-ggt ggg ccg agt gga att-3′
	Antisense	5′-ttg atg tgc ctg aga acg aag t-3′
	Probe	5′-ctg cct gcg cta ccg aga gga cct-3′
MRP2	Sense	5′-tgc agc ctc cat aac cat gag-3′
	Antisense	5′-ctt cgt ctt cct tca ggc tat tca-3′
	Probe	5′-cag ctt tcg tcg aac act tag ccg ca-3′
MRP3	Sense	5′-gcc atc gac ctg gag act ga-3′
	Antisense	5′-gac cct ggt gta gtc cat gat agt g-3′
	Probe	5′-cat ccg cac cca gtt tga tac ctg cac-3′
MRP4	Sense	5′-aag tga aca acc tcc agt tcc ag-3′
	Antisense	5′-ggc tct cca gag cac cat ct-3′
	Probe	5′-caa acc gaa gac tct gag aag gta cga ttc ct-3′
MRP5	Sense	5′-tga aag cca ttc gag gag ttg-3′
	Antisense	5′-cgg aaa agc tcg tca tgc a-3′
	Probe	5′-ctc gca gcg tgc cct tga caa ag-3′
MRP6	Sense	5′-aga cac ggt tga cgt gga cat-3′
	Antisense	5′-gct gac ctc cag gag tcc aa-3′
	Probe	5′-cca gac aaa ctc cgg tcc ctg ctg at-3′
MDR1	Sense	5′-ggc aaa gaa ata aag cga ctg aa-3′
	Antisense	5′-ggc tgt tgt ctc cat agg caa t-3′
	Probe	5′-cgt gtc cca gga gcc cat cct gt-3′
BSEP	Sense	5′-aca tgc ttg cga gga cct tta-3′
	Antisense	5′-gga ggt tcg tgc acc agg ta-3′
	Probe	5′-cca tcc ggc aac gct cca agt ct-3′

BSEP indicates bile salt export pump; MDR, multidrug resistance protein; and MRP, multidrug resistance–associated protein.

Double staining was detected with confocal laser scanning microscopy (a Zeiss 410 inverted laser scan microscope).

Isolation of Nuclear Extracts. HSCs were washed twice with ice-cold PBS and harvested in 500 μL of PBS. Nuclear extracts were isolated as described previously with 0.25% Nonidet P-40.[25]

Electrophoretic Mobility Shift Assay (EMSA). EMSAs were performed as described previously[25] with 5 μg of nuclear proteins in a total volume of 15 μL. The mdr-κB probe used, 5′gat cct ggg gaa ttc cag ctc-3′ with the NF-κB site underlined, has been published before.[26] Competition was performed with excess nonlabeled radioactive oligonucleotide. The reaction mixture was analyzed on a 4% polyacrylamide gel.

Immunocytochemistry. Analyses of active caspase-3

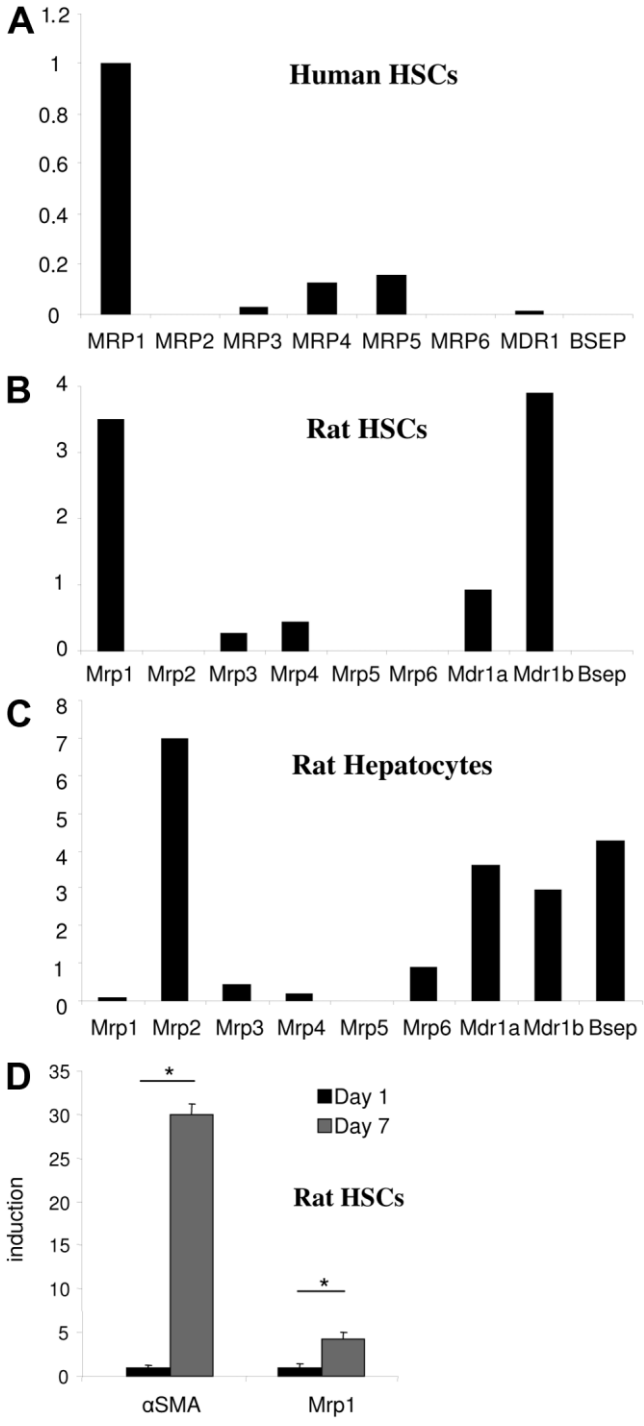

Fig. 1. Activated human and rat HSCs show high expression of Mrp1. (A-C) Relative mRNA expression levels were normalized to 18S. The human MRP1 $\delta\delta$Ct value was set to 1; for other genes and cell types, the $\delta\delta$Ct values were compared to human MRP1. Data are expressed as means $\pm$ standard deviation. MRP1/Mrp1 mRNA was abundant in activated HSCs, whereas MRP2/Mrp2, MRP6/Mrp6, and BSEP/Bsep were undetectable in HSCs but present in hepatocytes. Mrp5 was absent in rat HSCs and in hepatocytes, but not in human HSCs. Mrp3 and Mrp4 levels were comparable in HSCs and hepatocytes. Mdr1a and Mdr1b were prominently expressed in rat HSCs and hepatocytes. However, MDR1 expression in human HSCs was low. (D) Freshly isolated HSCs were cultured on plastic for 1 and 7 days. As expected, stellate cell activation was associated with the induction of α-SMA mRNA expression ($P < 0.05$). Expression of Mrp1 also increased 4-fold upon the activation of quiescent stellate cells ($P < 0.05$). α-SMA indicates α-smooth muscle actin; Bsep, bile salt export pump; HSC, hepatic stellate cell; Mdr, multidrug resistance protein; mRNA, messenger RNA; and Mrp, multidrug resistance–associated protein.

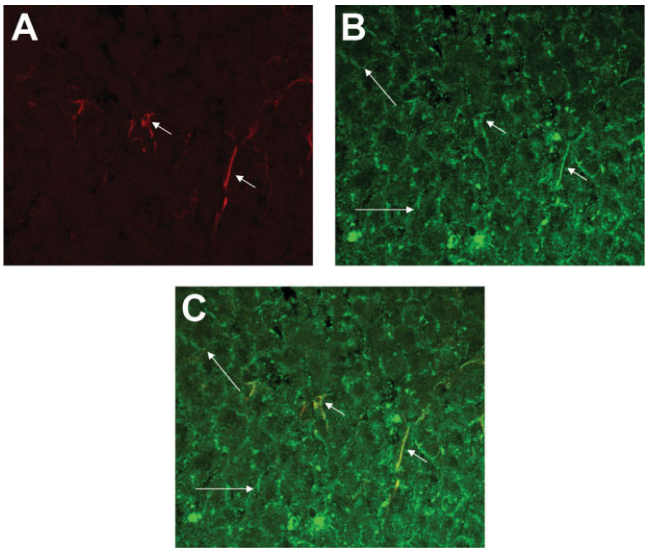

Fig. 2. HSCs express Mrp1 in CCl_4-treated rats. Double immunofluorescence staining for desmin, a marker of (A) HSCs and (C) Mrp1 in rats treated with CCl_4 for 2 weeks. (A) Desmin-positive HSCs (red labeling, short arrow). (B) Mrp1-positive HSC (green labeling, short arrow) and a specific focal membranous staining pattern of Mrp1 in hepatocytes (green labeling, long arrow). (C) Merged image: desmin-positive HSC cells are also Mrp1-positive (yellow labeling, short arrow). Mrp1 was also expressed in the basolateral membrane of hepatocytes, serving as an internal positive control (only green labeling, long arrow). CCl_4 indicates carbon tetrachloride; HSC, hepatic stellate cell; and Mrp, multidrug resistance–associated protein.

and α-smooth muscle actin were performed on cells cultured on coverslips. Coverslips were washed in PBS and fixed in 4% paraformaldehyde for 10 minutes. Coverslips were then stored in 0.1% PBS at 4°C. Prior to staining, cells were washed in PBS, incubated in PBS containing 1% Triton-X100 for 5 minutes, and washed twice with PBS. Primary antibodies against active caspase-3 (rabbit polyclonal antibody from Cell Signaling Technology, Beverly, MA) and α-smooth muscle actin (mouse monoclonal antibody from Sigma Aldrich) were used at a dilution of 1:100 and 1:1000, respectively, in 0.5% bovine serum albumin/PBS for 2 hours. Horseradish peroxidase–conjugated goat-anti-rabbit immunoglobulin (fluorescent conjugate; Invitrogen) and horseradish peroxidase–conjugated goat-anti-mouse immunoglobulin (fluorescent conjugate; Invitrogen) were used as secondary antibodies at a dilution of 1:400 for 1 hour. Coverslips were subsequently washed with PBS and mounted in the fluorescent mounting medium S3023 (Dako). All slides were evaluated on a Leica confocal laser scanning microscope.

Flow Cytometry and Transporter Efflux Assay. HSCs were incubated with either 200 ng/mL rhodamine 123 (Molecular Probes) as an Mdr substrate or 0.1 μmol/L Cell Tracker green 5-chloromethylfluorescein diacetate (CMFDA; Invitrogen) as an Mrp substrate for 30 minutes, with and without their corresponding Mdr and Mrp inhibitors. Cells were washed and exposed to either Mdr or Mrp inhibitors without Mdr/Mrp substrate for another 60 minutes. Cells were then trypsinized, harvested, and incubated for 15 minutes in ice-cold PBS. After incubation, 5000 cells per sample were counted, and viable cells were analyzed for rhodamine 123 or CMFDA fluorescence with a FACSCalibur instrument (BD Bioscience, Alphen aan de Rijn, the Netherlands).

Acridine Orange and Sytox Green Nuclear Staining. Cells were seeded in 12-well plates and treated as indicated. Apoptosis was shown by the determination of nuclear condensation assessed by acridine orange staining at 2.5 μg/mL (Sigma Aldrich). Necrosis was shown by Sytox green nucleic acid staining at 0.5 μmol/L (Invitro-

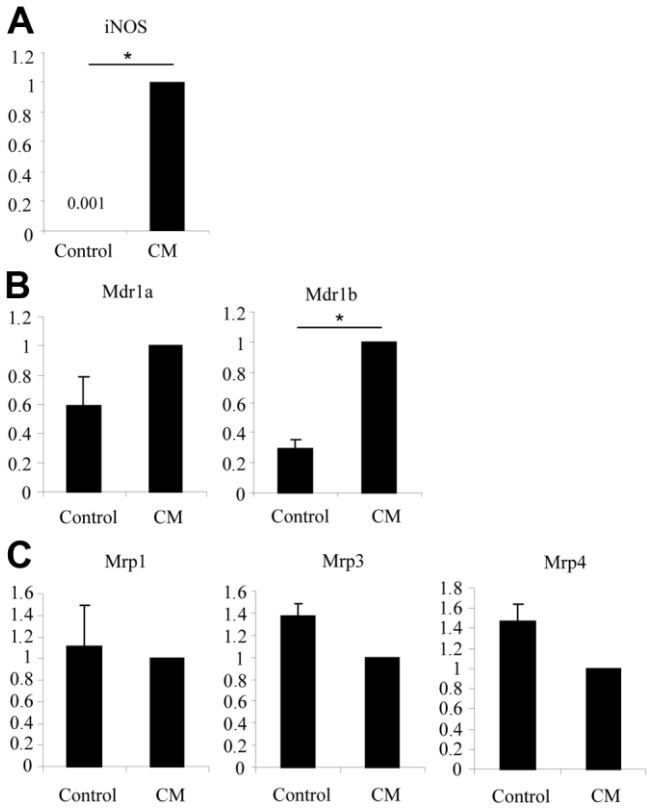

Fig. 3. Mdr1b is a cytokine-inducible, NF-κB–responsive gene. CM (tumor necrosis factor-α; 20 ng/mL; interleukin-1β, 10 ng/mL; and interferon-γ, 10 ng/mL) significantly induced the NF-κB–responsive gene iNOS. Mdr1b, like iNOS, was significantly induced by cytokines, unlike Mdr1a- and Mrp-type transporters. (A) Quantitative real-time PCR for iNOS. The numbers above the left bar represent the fold induction in comparison with CM-treated cells ($^*P < 0.05$). (B) Quantitative real-time PCR for Mdr1a and Mdr1b ($^*P < 0.05$). (C) Quantitative real-time PCR for Mrp1, Mrp3, and Mrp4 ($^*P < 0.05$). CM indicates cytokine mixture; iNOS, inducible nitric oxide synthase; Mdr, multidrug resistance protein; Mrp, multidrug resistance–associated protein; and PCR, polymerase chain reaction.

gen). Cells were monitored over time with an Olympus CKX41 microscope.

Statistical Analysis. Statistical analysis was performed with SPSS software, version 12.0. Results are expressed as the mean value and standard deviation. Statistical significance was calculated with a Student *t* test or a one-way analysis of variance with Bonferroni post hoc analysis for correction for multiple comparisons. A corrected P value < 0.05 was considered to be statistically significant.

Results

Activated Rat HSCs Have a Different ABC Transporter Messenger RNA (mRNA) Profile Than Hepatocytes. To determine whether activated rat HSCs express ABC transporters, we isolated mRNA from these cells and analyzed the expression of selected ABC transporter genes. We compared this to the ABC transporter expression profile of freshly isolated rat hepatocytes (Fig. 1B,C). Rat hepatocytes highly expressed ABC transporters that are active in bile formation, including Mrp2 and bile salt export pump (Bsep). As expected, the expression of these genes was low to undetectable in activated HSCs. In contrast, the expression of Mrp1 was particularly high in activated HSCs, and this expression was clearly associated with the activated phenotype, as Mrp1 expression increased upon the activation of quiescent stellate cells (Fig. 1D). In addition, comparable expression levels of Mdr1a, Mdr1b, Mrp3, and Mrp4 were observed in activated HSCs and hepatocytes. Expression of Mrp5 remained undetectable in both cell types (Fig. 1B,C).

The MRP Transporter Profile of Rat HSCs Is Similar to Human HSCs. MRP1/Mrp1 was the most prominent transporter in human HSCs and in rat HSCs (Fig. 1A,B). Expression levels of MRP3/Mrp3 and MRP4/Mrp4 were also similar in rat and human cells. However, MRP5 was expressed in human HSCs, whereas we were unable to detect Mrp5 in rat HSCs. MDR1 expression in human HSCs was low in comparison with Mdr1b expression in rat HSCs.

Mrp1 Is Present in HSCs in Experimental Fibrosis in Rats. Liver slices from rats exposed to CCl_4 for 2 weeks were stained for Mdr transporters, Mrp1, and Mrp3. Desmin and GFAP were used as markers for HSCs. Desmin-positive cells were also positive for Mrp1 (Fig. 2C). However, no overlap was found between desmin or GFAP with Mdr transporters or Mrp3 (data not shown).

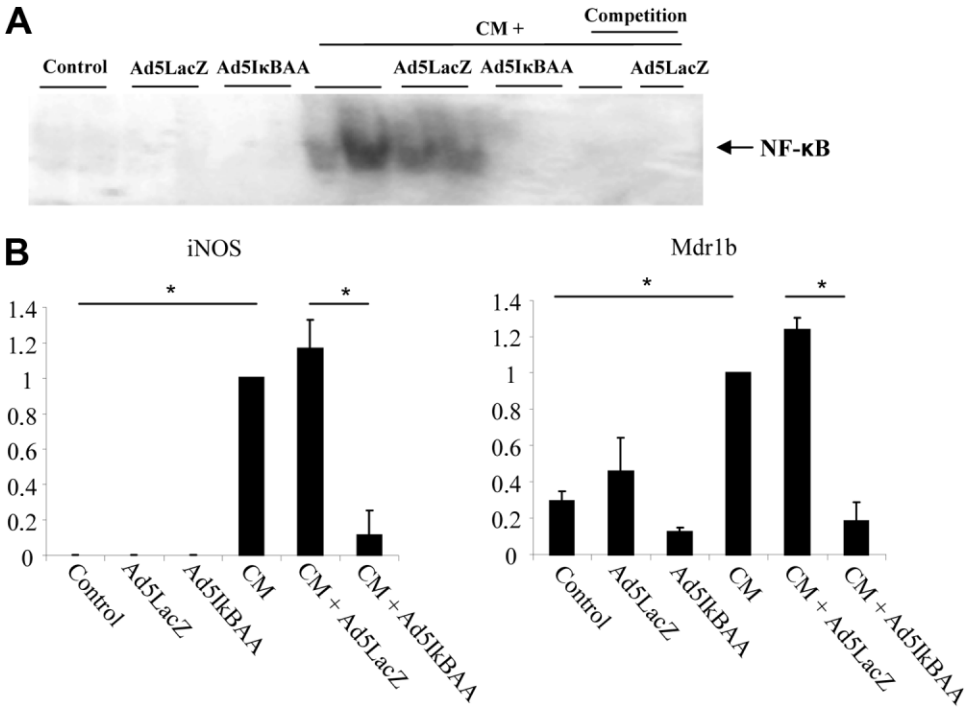

Fig. 4. NF-κB is not activated in untreated HSCs but is activated by cytokines, and iNOS and Mdr1b are NF-κB–responsive genes. (A) Electrophoretic mobility shift assay for NF-κB. In untreated activated HSCs, NF-κB was not activated. Cells exposed to a CM (tumor necrosis factor α, 20 ng/mL; interleukin-1β, 10 ng/mL; and interferon-γ, 10 ng/mL) for 6 hours demonstrated activation of NF-κB, which was abolished by the dominant negative NF-κB adenovirus. Competition with excess nonlabeled oligonucleotide completely abolished CM-induced NF-κB activation. (B) Quantitative real-time polymerase chain reaction, showing a significant reduction in iNOS and Mdr1b expression when NF-κB was inhibited (*$P <$ 0.05). CM indicates cytokine mixture; HSC, hepatic stellate cell; iNOS, inducible nitric oxide synthase; Mdr, multidrug resistance protein; and NF-κB, nuclear factor-κB.

Cytokines Induce Mdr1b mRNA Levels in Activated HSCs. The expression of several ABC transporters has been shown to be regulated during inflammation and/or oxidative stress. Therefore, we investigated whether cytokines could influence the expression level of the most prominent ABC transporters present in HSCs. Activated HSCs were exposed for 6 hours to a cytokine mixture (CM) consisting of 20 ng/mL mouse tumor necrosis factor α, 10 ng/mL human interleukin-1β, and 10 ng/mL rat interferon-γ. Total RNA was isolated. mRNA levels of the NF-κB–controlled gene inducible nitric oxide synthase (iNOS) were significantly increased in the CM-treated HSC cells (>1000-fold; Fig. 3A). Of the ABC transporter genes tested, only Mdr1b expression was significantly induced by CM treatment (4-fold; Fig. 3B,C).

Mdr1b Induction by Cytokines Is NF-κB–Dependent in Activated HSCs. Next, we determined whether cytokine-induced Mdr1b expression was dependent on NF-κB activation. Nuclei were isolated from activated HSCs that had been exposed to CM for 6 hours as well as untreated HSCs. EMSA analyses using these nuclear extracts and an NF-κB–specific DNA probe showed that the nuclei of CM-treated HSCs contained NF-κB, whereas this transcription factor was absent in control nuclei (Fig. 4A). Cotreatment with a dominant negative NF-κB adenovirus (Ad5IκBAA) abolished both the level of nuclear NF-κB (Fig. 4A) and the CM-dependent Mdr1b expression in CM-treated HSCs (Fig. 4B). Infection with Ad5LacZ did not decrease nuclear NF-κB levels or Mdr1b mRNA expression levels (Fig. 4A,B). The effect of CM treatment and NF-κB inhibition on Mdr1b expression was paralleled by iNOS, a well-known target gene of NF-κB (Fig. 4B).

Oxidative Stress Is Not a Major Regulator of Mrp and Mdr mRNA Expression in Activated HSCs. To determine whether other disease conditions affect the ABC transporter expression in HSCs, we studied the effect of different inducers of oxidative stress on their tran-

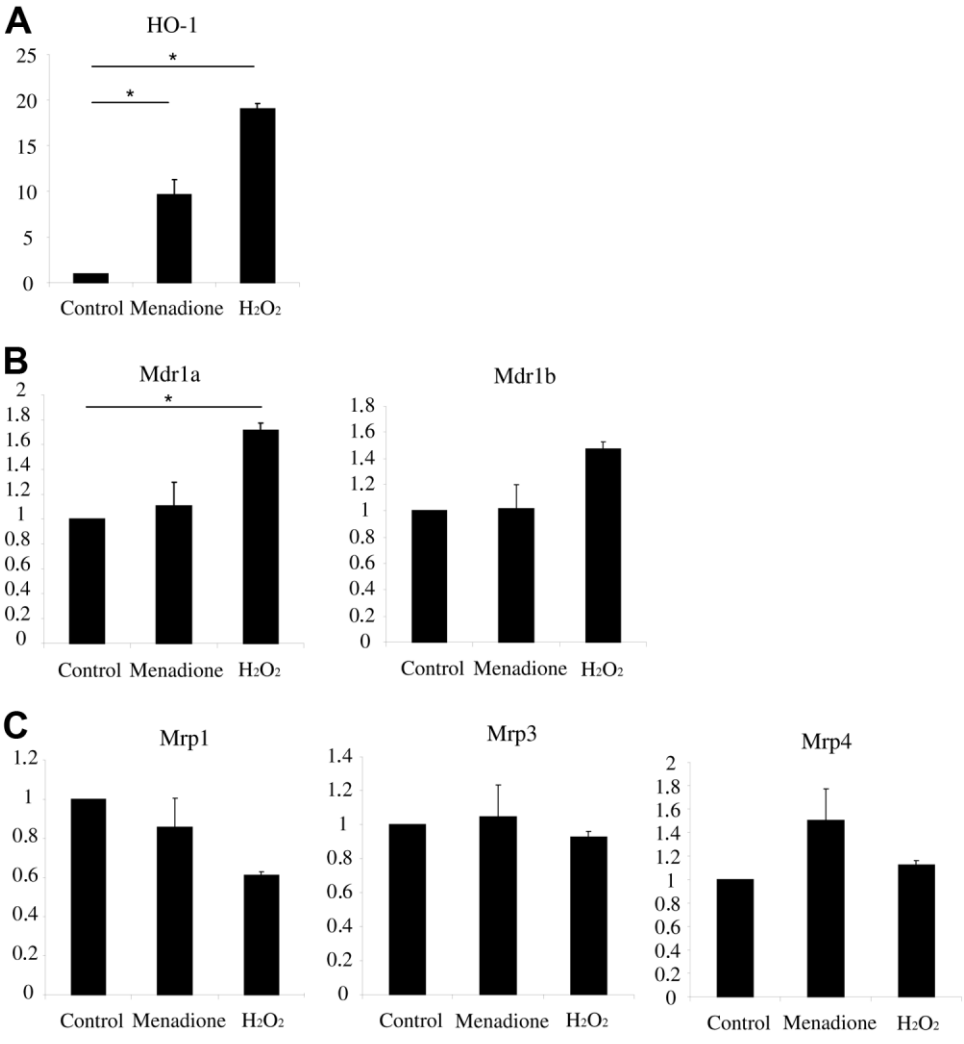

Fig. 5. Oxidative stress is not a major regulator of ATP-binding cassette transporter expression. The oxidative stress–responsive gene HO-1 was significantly induced by 10 μmol/L menadione or 200 μmol/L H_2O_2. Mdr1a expression was slightly increased by hydrogen peroxide, whereas other Mrp and Mdr transporters were not significantly altered by exposure to reactive oxygen species. (A) Quantitative real-time PCR for HO-1 (*$P < 0.05$). (B) Quantitative real-time PCR for Mdr1a and Mdr1b (*$P < 0.05$). (C) Quantitative real-time PCR for Mrp1, Mrp3, and Mrp4 (*$P < 0.05$). H_2O_2 indicates hydrogen peroxide; HO-1, heme-oxygenase-1; Mdr, multidrug resistance protein; Mrp, multidrug resistance–associated protein; and PCR, polymerase chain reaction.

scription levels. HSCs were exposed for 6 hours to either 200 μmol/L hydrogen peroxide or 10 μmol/L menadione (an intracellular generator of superoxide anions). Under these conditions, expression of a typical marker gene for oxidative stress, heme-oxygenase-1 (HO-1), was significantly induced, albeit at variable levels (Fig. 5A). In contrast, only Mdr1a was slightly induced with hydrogen peroxide (Fig. 5B). Mdr1b and Mrp transporters were not significantly altered by exposure to exogenous reactive oxygen species (Fig. 5B,C).

Both Mdr-Type and Mrp-Type Transporters Are Functional Efflux Transporters in Activated HSCs. To determine whether Mdr transporters not only are present but also function as efflux transporters, we used a flow cytometry efflux assay. We exposed cells to the Mdr substrate rhodamine 123, with or without 50 μmol/L verapamil or 50 μmol/L PSC-833 as Mdr inhibitors. Cells incubated with both rhodamine 123 and either of the Mdr inhibitors showed significantly higher fluorescence content than cells incubated with rhodamine 123 alone (Fig. 6A). These data demonstrate that the Mdr transporters which are present also function as efflux transporters and that both verapamil and PSC-833 are efficient Mdr inhibitors.

To determine whether Mrp transporters not only are present but also function as efflux transporters, we used a flow cytometry efflux essay. We exposed cells to the Mrp substrate CMFDA with and without 50 μmol/L MK571 as an Mrp inhibitor. Cells incubated with both CMFDA and MK571 showed a significantly higher fluorescence content than cells incubated with MK571 alone (Fig. 6B). This demonstrates that the Mrp transporters which are present also function as efflux transporters and that MK571 is an efficient Mrp inhibitor.

Mrp-Type Transporters Are Essential for the Viability of Activated HSCs. In order to determine the function of ABC transporter expression in activated HSCs, we used various inhibitors of ABC transporters and evaluated their effects on HSC viability. First, we tested whether inhibition of Mrp function by MK571 induced apoptosis of activated HSCs (Fig. 7; red: αSMA cytoskeleton, green: active caspase-3). Gliotoxin, which is known to induce HSC apoptosis, was used as a positive control. Indeed, gliotoxin induced typical characteristics of apoptosis in HSCs, such as condensed nuclei (Fig. 7B) and activation of caspase-3 (Fig. 7E), which was not observed in untreated cells (Fig. 7A,D). In contrast, MK571, a selective inhibitor of Mrp function, did not cause nuclear condensation (Fig. 7C) or activation of caspase-3 (Fig. 7F). However, MK571 treatment clearly affected HSC cell morphology and disrupted the α-smooth muscle actin cytoskeleton (Fig. 7F). Therefore, we tested whether MK571 induces HSC necrosis. Indeed, over 50% of the HSCs became necrotic, as early as 6 hours after exposure to 50 μmol/L MK571 (Fig. 8B). After an additional 18 hours, no viable HSCs were detected. All cells contained Sytox green fluorescent nuclei, which is indicative of necrotic

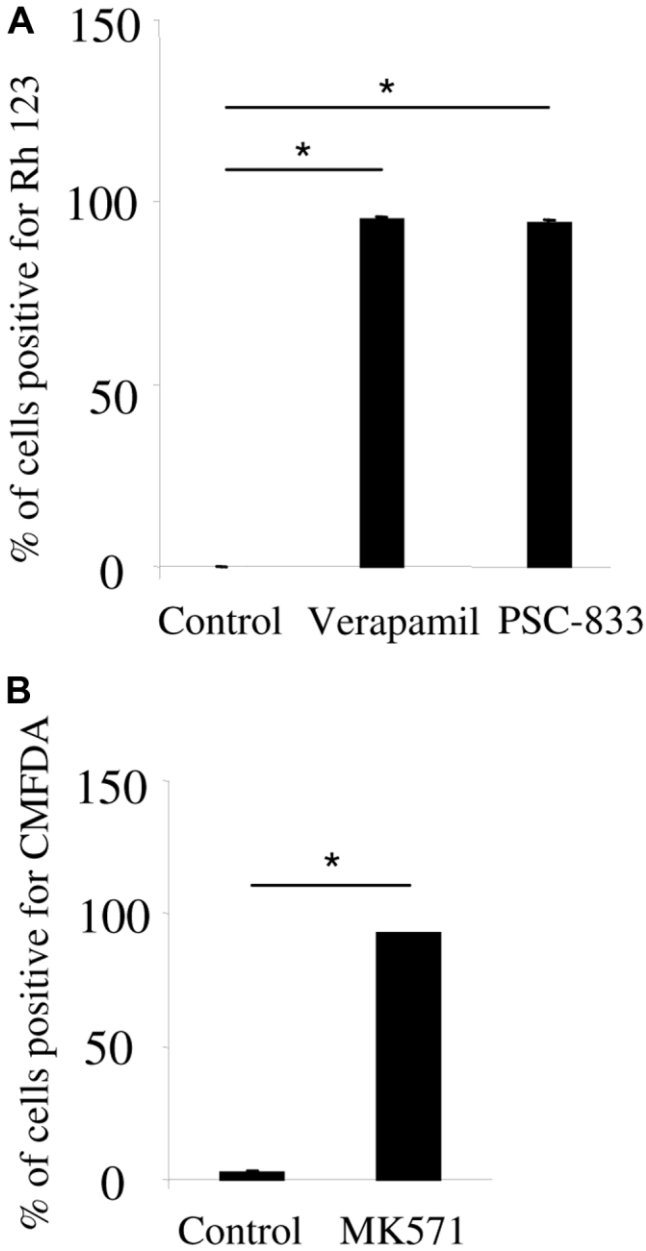

Fig. 6. Multidrug resistance protein and multidrug resistance–associated protein transporters are functional efflux transporters in activated hepatic stellate cells. (A) Cells were exposed to 200 ng/mL rhodamine 123 with or without either 50 μmol/L verapamil or 50 μmol/L PSC-833. Of the cells exposed to rhodamine 123 and verapamil or PSC-833, 95.5% and 94.2%, respectively, were considered positive for rhodamine 123 fluorescence versus 0.2% of cells exposed to rhodamine 123 alone (*$P < 0.05$). (B) Cells were exposed to 0.1 μmol/L CMFDA with or without 50 μmol/L MK571. Of the cells exposed to CMFDA and MK571, 92.4% were considered positive for CMFDA fluorescence versus 3.0% of cells exposed to CMFDA alone (*$P < 0.05$). Only viable cells were included in the analysis. CMFDA indicates 5-chloromethylfluorescein diacetate;

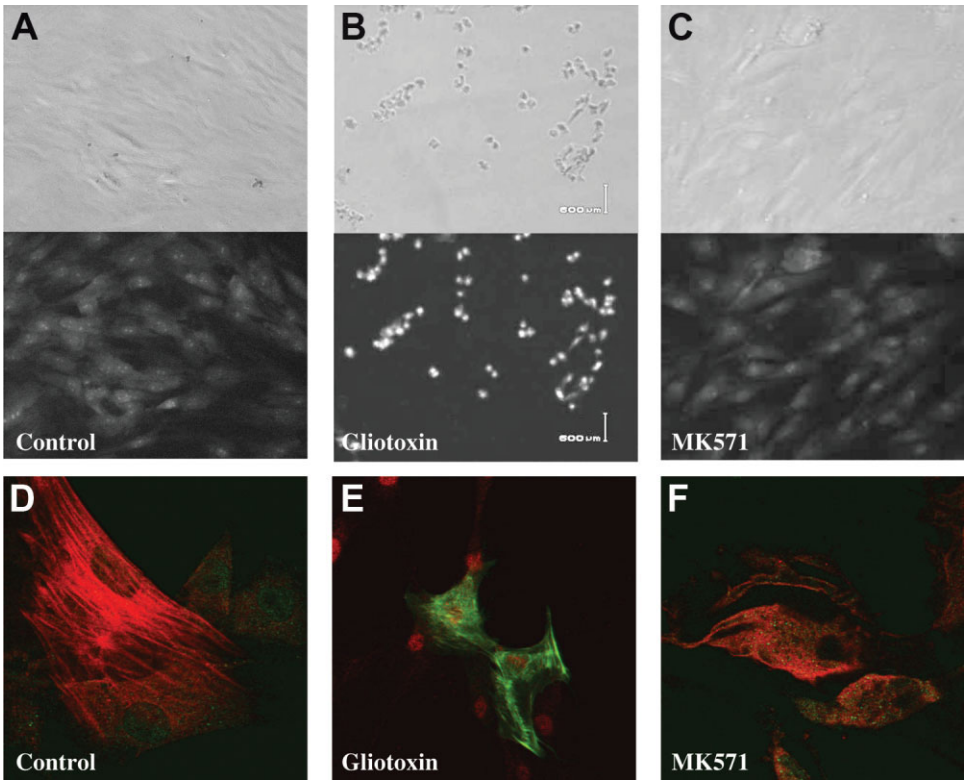

Fig. 7. MK571 does not cause apoptosis in activated hepatic stellate cells. Cells were exposed to 50 μmol/L MK571 for 6 hours. At concentrations of 50 μmol/L, MK571 did not induce apoptosis as judged by nuclear morphology with (C) acridine orange staining and (F) a lack of activated caspase-3 staining (green), but cell morphology was changed in cells treated with MK571 for 6 hours as judged by (F) staining for α-smooth muscle actin (red). (B,E) Gliotoxin was used as a positive control for hepatic stellate cell apoptosis. (B) Nuclear condensation as a marker for apoptosis. (E) The disruption of the actin cytoskeleton (red) and staining of active caspase-3 (green) as a marker for apoptosis is shown. (A,D) Untreated cells are depicted.

cell death (Fig. 8C). In parallel experiments, 50 μmol/L MK571 did not significantly affect hepatocyte viability (data not shown). The Mdr inhibitors verapamil (50 μmol/L) and PSC-833 (50 μmol/L) caused neither apoptotic nor necrotic death of activated HSCs (Fig. 9A-C).

Discussion

Although the function of many ABC transporters has been studied extensively in hepatocytes[12] and HPCs,[14] their presence and function in activated HSCs have never been investigated. In this study, we showed that activated rat HSCs expressed Mrp1, Mrp3, Mrp4, Mdr1a, and Mdr1b mRNA. This expression pattern is similar to that seen in HPCs.[14,27] Activated human HSCs also expressed MRP1, MRP3, MRP4, and, unlike rat HSCs, MRP5. Expression of MDR1 in human HSCs was low compared to rat HSCs. Neither rat nor human HSCs expressed Mrp2/MRP2, Mrp6/MRP6, or Bsep/BSEP, which are prominent transporters in rat hepatocytes. In comparison with rat hepatocytes, Mrp1 expression was high in both rat and human HSCs. In immunohistochemical double staining of liver sections from rats treated with CCl_4 for 2 weeks, desmin-positive HSCs also stained for Mrp1. However, no overlap could be found between desmin or GFAP with Mrp3 or Mdr transporters.

High expression of Mrp-type and Mdr-type transporters is associated with enhanced resistance to cell death.[11] Activated and proliferating HSCs may use these trans-

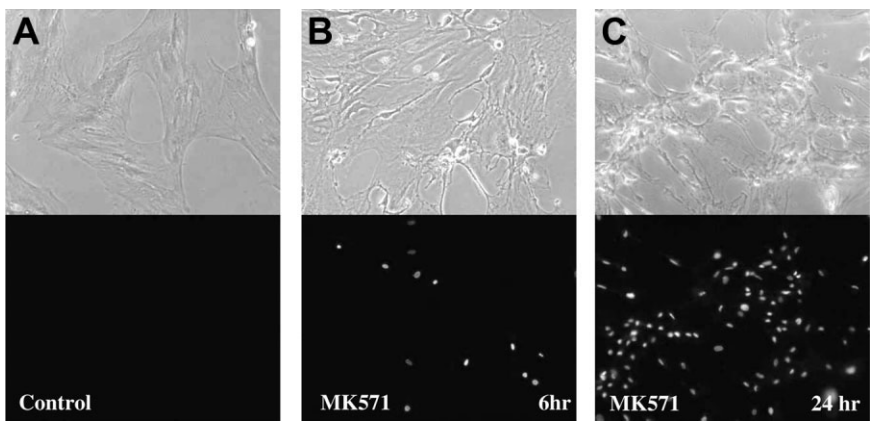

Fig. 8. MK571 induces necrosis in activated hepatic stellate cells. Cells were exposed to 50 μmol/L MK571 for the indicated time. (B,C) Sytox green staining, indicative of necrotic cell death, clearly demonstrated that inhibition of multidrug resistance–associated protein–type transports induced necrotic cell death in activated stellate cells. (A) Untreated cells are depicted.

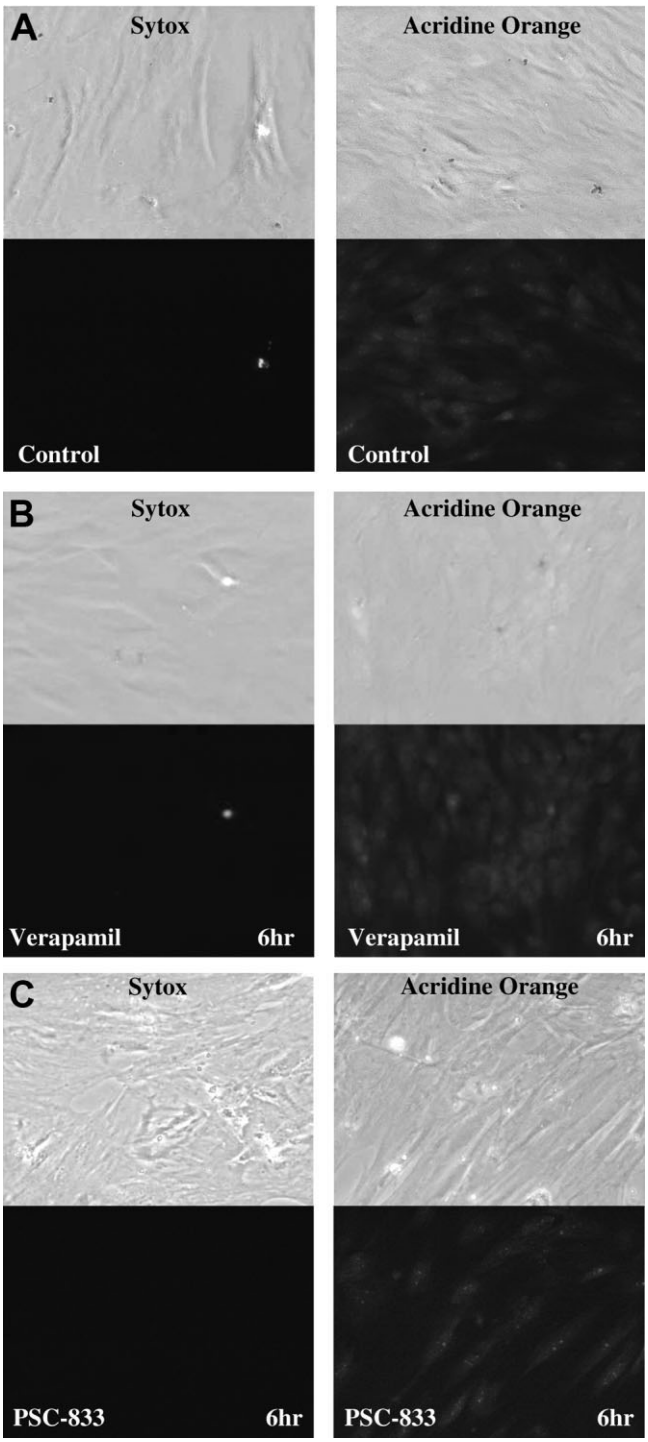

Fig. 9. The multidrug resistance protein inhibitors verapamil and PSC-833 do not cause cell death in activated hepatic stellate cells. Cells were (A) untreated or exposed to (B) 50 μmol/L verapamil or (C) 50 μmol/L PSC-833 for 6 hours. Neither verapamil nor PSC-833 induced necrosis, as judged by Sytox green nuclear staining, or apoptosis, as judged by nuclear morphology with acridine orange staining.

porters to clear their cells of exogenous and endogenous toxic substrates generated in the injured liver, endowing these cells with a survival advantage in a hostile environment. We showed that inhibition of Mrp-type transporters with MK571 induced cell death in activated HSCs, which could be caused by the accumulation of a toxic endogenous Mrp substrate. As reported for other cell types,[28,29] the Mdr1b mRNA level in activated stellate cells was increased by cytokines in an NF-κB–dependent manner. No significant changes were observed in Mrp1, Mrp3, and Mrp4 mRNA levels of activated stellate cells exposed to cytokines. The Mrp regulatory response to cytokines varies between cell types. Lee and Piquette-Miller[30] described an increase in Mrp3 expression in Huh7 cells in response to cytokine exposure. Tumor necrosis factor α–treated HepG2 cells showed a decrease in expression of Mrp1 and Mrp3, whereas Mrp1 could be induced with interleukin-1β. We used a cytokine mixture to mimic the cytokine profile observed under inflammatory conditions.[31] Oxidative stress did not change the expression of Mrp-type ABC transporters, and hydrogen peroxide only slightly increased Mdr1a expression. Little is known about the regulation of Mrp-type and Mdr-type transporters by oxidative stress. Our results could imply that reactive oxygen species, at least in stellate cells, are not major regulators of these transporters. Stellate cells do respond to reactive oxygen species, as demonstrated by the increased expression of the oxidative stress–responsive gene HO-1. Yet, there could be a role for Mrp-type transporters in the detoxification of reactive oxygen species. The expression of Mrp-type transporters in stellate cells was significant, and these transporters are known to be involved in the efflux of glutathione conjugates generated in the detoxification of reactive oxygen species. Furthermore, Hammond et al.[32] reported on Mrp1-dependent efflux of reduced glutathione in several cell types. According to this study, Mrp1-dependent efflux of reduced glutathione is a prerequisite for apoptosis. The interrelationship between reactive oxygen species, glutathione status, and cell death is currently being investigated in our laboratory.

In comparison with activated stellate cells, Mrp1 mRNA expression is low in hepatocytes. However, hepatocytes express other Mrp-type efflux pumps, such as Mrp3 and Mrp2. The latter is hepatocyte-specific in the liver, localized at the canalicular membrane and plays an important role in bile formation. The differential expression and/or localization of Mrp-type transporters in hepatocytes and activated stellate cells may explain the differential sensitivity to inhibition of Mrp function.

Although culture-activated rat HSCs expressed Mdr transporters that were functional as efflux transporters, we could not detect Mdr transporters in immunohistochemical double staining in liver sections of CCl_4-treated rats. Also, the Mdr inhibitors verapamil and PSC-833 (a cyclosporin A analogue) did not affect cell death in culture-

activated HSCs. Although Mdr transporters did not seem as important as Mrp transporters for the survival of activated HSCs in our *in vitro* conditions, they might play a role *in vivo* by transporting exogenous toxins or xenobiotics.

In conclusion, these results demonstrate that activated HSCs express high levels of several Mrp-type and Mdr-type transporters under conditions prevailing in the chronically injured liver, which may enable these cells to survive in this hostile environment. We hypothesize that MK571 induces cell death in HSCs by inhibiting Mrp1. Although the Mrp substrate involved in this process has not been elucidated, we suggest that inhibiting Mrp1 function in chronic liver disease may cause cell death in activated HSCs without affecting hepatocytes, thereby presenting an interesting target for drug therapy aimed at preventing or reversing liver fibrosis and cirrhosis. Because the activation and proliferation of HSCs are the common denominator of most chronic liver diseases, this strategy holds great promise for the treatment of chronic liver diseases, regardless of the etiology.[6]

References

1. Schoemaker MH, Moshage H. Defying death: the hepatocyte's survival kit. Clin Sci (Lond) 2004;107:13-25.
2. Kaplowitz N. Mechanisms of liver cell injury. J Hepatol 2000;32(Suppl): 39-47.
3. Sell S. Heterogeneity and plasticity of hepatocyte lineage cells. HEPATOLOGY 2001;33:738-750.
4. Friedman SL. Liver fibrosis—from bench to bedside. J Hepatol 2003; 38(Suppl 1):S38-S53.
5. Libbrecht L, Roskams T. Hepatic progenitor cells in human liver diseases. Semin Cell Dev Biol 2002;13:389-396.
6. Bataller R, Brenner DA. Liver fibrosis. J Clin Invest 2005;115:209-218.
7. Jaeschke H, Gores GJ, Cederbaum AI, Hinson JA, Pessayre D, Lemasters JJ. Mechanisms of hepatotoxicity. Toxicol Sci 2002;65:166-176.
8. Friedman SL. Molecular regulation of hepatic fibrosis, an integrated cellular response to tissue injury. J Biol Chem 2000;275:2247-2250.
9. Svegliati-Baroni G, Ridolfi F, Hannivoort R, Saccomanno S, Homan M, De MS, et al. Bile acids induce hepatic stellate cell proliferation via activation of the epidermal growth factor receptor. Gastroenterology 2005;128: 1042-1055.
10. Elsharkawy AM, Oakley F, Mann DA. The role and regulation of hepatic stellate cell apoptosis in reversal of liver fibrosis. Apoptosis 2005;10:927-939.
11. Borst P, Elferink RO. Mammalian ABC transporters in health and disease. Annu Rev Biochem 2002;71:537-592.
12. Faber KN, Muller M, Jansen PL. Drug transport proteins in the liver. Adv Drug Deliv Rev 2003;55:107-124.
13. Higgins CF. Multiple molecular mechanisms for multidrug resistance transporters. Nature 2007;446:749-757.
14. Ros JE, Roskams TA, Geuken M, Havinga R, Splinter PL, Petersen BE, et al. ATP binding cassette transporter gene expression in rat liver progenitor cells. Gut 2003;52:1060-1067.
15. Vander BS, Libbrecht L, Katoonizadeh A, van PJ, Cassiman D, Nevens F, et al. Breast cancer resistance protein (BCRP/ABCG2) is expressed by progenitor cells/reactive ductules and hepatocytes and its expression pattern is influenced by disease etiology and species type: possible functional consequences. J Histochem Cytochem 2006;54:1051-1059.
16. Cassiman D, Denef C, Desmet VJ, Roskams T. Human and rat hepatic stellate cells express neurotrophins and neurotrophin receptors. HEPATOLOGY 2001;33:148-158.
17. Moshage H, Casini A, Lieber CS. Acetaldehyde selectively stimulates collagen production in cultured rat liver fat-storing cells but not in hepatocytes. HEPATOLOGY 1990;12(pt 1):511-518.
18. Wright MC, Issa R, Smart DE, Trim N, Murray GI, Primrose JN, et al. Gliotoxin stimulates the apoptosis of human and rat hepatic stellate cells and enhances the resolution of liver fibrosis in rats. Gastroenterology 2001; 121:685-698.
19. Schoemaker MH, Gommans WM, Conde de la RL, Homan M, Klok P, Trautwein C, et al. Resistance of rat hepatocytes against bile acid-induced apoptosis in cholestatic liver injury is due to nuclear factor-kappa B activation. J Hepatol 2003;39:153-161.
20. Gekeler V, Ise W, Sanders KH, Ulrich WR, Beck J. The leukotriene LTD4 receptor antagonist MK571 specifically modulates MRP associated multidrug resistance. Biochem Biophys Res Commun 1995;208:345-352.
21. Mickley LA, Bates SE, Richert ND, Currier S, Tanaka S, Foss F, et al. Modulation of the expression of a multidrug resistance gene (mdr-1/P-glycoprotein) by differentiating agents. J Biol Chem 1989;264:18031-18040.
22. Boesch D, Gaveriaux C, Jachez B, Pourtier-Manzanedo A, Bollinger P, Loor F. In vivo circumvention of P-glycoprotein-mediated multidrug resistance of tumor cells with SDZ PSC 833. Cancer Res 1991;51:4226-4233.
23. Iimuro Y, Nishiura T, Hellerbrand C, Behrns KE, Schoonhoven R, Grisham JW, et al. NFkappaB prevents apoptosis and liver dysfunction during liver regeneration. J Clin Invest 1998;101:802-811.
24. Blokzijl H, Vander BS, Bok LI, Libbrecht L, Geuken M, van den Heuvel FA, et al. Decreased P-glycoprotein (P-gp/MDR1) expression in inflamed human intestinal epithelium is independent of PXR protein levels. Inflamm Bowel Dis 2007;13:710-720.
25. Tuyt LM, Bregman K, Lummen C, Dokter WH, Vellenga E. Differential binding activity of the transcription factor LIL-STAT in immature and differentiated normal and leukemic myeloid cells. Blood 1998;92:1364-1373.
26. Zhou G, Kuo MT. NF-kappaB-mediated induction of mdr1b expression by insulin in rat hepatoma cells. J Biol Chem 1997;272:15174-15183.
27. Ros JE, Libbrecht L, Geuken M, Jansen PL, Roskams TA. High expression of MDR1, MRP1, and MRP3 in the hepatic progenitor cell compartment and hepatocytes in severe human liver disease. J Pathol 2003;200:553-560.
28. Deng L, Lin-Lee YC, Claret FX, Kuo MT. 2-Acetylaminofluorene upregulates rat mdr1b expression through generating reactive oxygen species that activate NF-kappa B pathway. J Biol Chem 2001;276:413-420.
29. Ros JE, Schuetz JD, Geuken M, Streetz K, Moshage H, Kuipers F, et al. Induction of Mdr1b expression by tumor necrosis factor-alpha in rat liver cells is independent of p53 but requires NF-kappaB signaling. HEPATOLOGY 2001;33:1425-1431.
30. Lee G, Piquette-Miller M. Cytokines alter the expression and activity of the multidrug resistance transporters in human hepatoma cell lines; analysis using RT-PCR and cDNA microarrays. J Pharm Sci 2003;92:2152-2163.
31. Schoemaker MH, Ros JE, Homan M, Trautwein C, Liston P, Poelstra K, et al. Cytokine regulation of pro- and anti-apoptotic genes in rat hepatocytes: NF-kappaB-regulated inhibitor of apoptosis protein 2 (cIAP2) prevents apoptosis. J Hepatol 2002;36:742-750.
32. Hammond CL, Marchan R, Krance SM, Ballatori N. Glutathione export during apoptosis requires functional multidrug resistance-associated proteins. J Biol Chem 2007;282:14337-14347.