

Up-regulation and Cytoprotective Role of Epithelial Multidrug Resistance-associated Protein 1 in Inflammatory Bowel Disease^{*[S]}

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MRP1 (multidrug resistance-associated protein 1) is well known for its role in providing multidrug resistance to cancer cells. In addition, MRP1 has been associated with both pro- and anti-inflammatory functions in nonmalignant cells. The pro-inflammatory function is evident from the fact that MRP1 is a high affinity transporter for cysteinyl-leukotriene C₄ (LTC₄), a lipid mediator of inflammation. It remains unexplained, however, why the absence of Mrp1 leads to increased intestinal epithelial damage in mice treated with dextran-sodium sulfate, a model for inflammatory bowel disease (IBD). We found that MRP1 expression is induced in the inflamed intestine of IBD patients, e.g. Crohn disease and ulcerative colitis. Increased MRP1 expression was detected at the basolateral membrane of intestinal epithelial cells. To study a putative role for MRP1 in protecting epithelial cells against inflammatory cues, we manipulated MRP1 levels in human epithelial DLD-1 cells and exposed these cells to cytokines and anti-Fas. Inhibition of MRP1 (by MK571 or RNA interference) resulted in increased cytokine- and anti-Fas-induced apoptosis of DLD-1 cells. Opposite effects, e.g. protection of DLD-1 cells against cytokine- and anti-Fas-induced apoptosis, were observed after recombinant MRP1 overexpression. Inhibition of LTC₄ synthesis reduced anti-Fas-induced apoptosis when MRP1 function was blocked, suggesting that LTC₄ is the pro-apoptotic compound exported by epithelial MRP1 during inflammation. These data show that MRP1 protects intestinal epithelial cells against inflammation-induced apoptotic cell death and provides a functional role for MRP1 in the inflamed intestinal epithelium of IBD patients.

Inflammatory bowel disease (IBD)³ is characterized by chronic inflammation of the gastrointestinal tract and manifests as ulcer-

ative colitis (UC) or Crohn disease (CD) (1). The pathophysiology of IBD is characterized by a highly activated state of the mucosal immune system and excessive mucosal damage (2). In recent years, important progress has been made in identifying and characterizing susceptibility genes for IBD (3). The (putative) functions of the proteins they encode corroborate the notion that the primary cause for the development of IBD originates from a dysregulated immune response to commensal intestinal bacteria, defects in mucosal barrier function, and/or bacterial clearance.

Irrespective of the genetic cause, severe and continuous inflammation causes damage to the intestinal epithelium that may strongly affect its absorptive and secretory functions as well as its protective role against toxic compounds. Cytoprotection is provided by ATP-binding cassette (ABC) transporters that are specialized in exporting toxic compounds of foreign or endogenous origin (4). P-gp/MDR1 (ABCB1), well known for its adverse role in protecting cancer cells against anti-cancer drugs (5), is also expressed in the healthy intestinal epithelium (6). Absence of *Mdr1a* in transgenic mice results in the spontaneous development of colitis (7). In line with this observation, specific single nucleotide polymorphisms in the *ABCB1* gene have been reported to be associated with CD (8–10). Moreover, we recently showed that MDR1 expression is strongly reduced in the inflamed intestine of IBD patients, which may further aggravate the disease (11).

MRP1 (multidrug resistance-associated protein 1, encoded by the *ABCC1* gene) also plays a role in inflammatory responses. It transports glutathione and substrates that are conjugated to reduced glutathione, glucuronide, or sulfate as part of the detoxification machinery of (cancer) cells (12, 13). The glutathione conjugate leukotriene C₄ (LTC₄) has been identified as an endogenous, high affinity substrate for MRP1 (14, 15). Leukotrienes are important lipid mediators of inflammatory responses and have been implicated in the pathophysiology of both acute and chronic inflammatory diseases, including IBD (14, 16, 17). The role of Mrp1 in the inflammatory signaling pathway is evident from studies with Mrp1^{-/-} knock-out mice that show a strongly reduced response to arachidonic acid-induced inflammatory stimuli as measured by decreased ear edema and vascular permeability (18). Relevant for IBD, however, it was shown that intestinal damage

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^[S] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Table S1 and Fig. 1.

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³ The abbreviations used are: IBD, inflammatory bowel disease; UC, ulcerative colitis; CD, Crohn disease; LTC₄, leukotriene C₄; ABC transporters, ATP-binding cassette transporters; MRP, multidrug resistance-associated pro-

tein; PARP, poly(ADP-ribose)polymerase; siRNA, short interfering RNA; TNF- α , tumor necrosis factor- α ; GFP, green fluorescent protein.

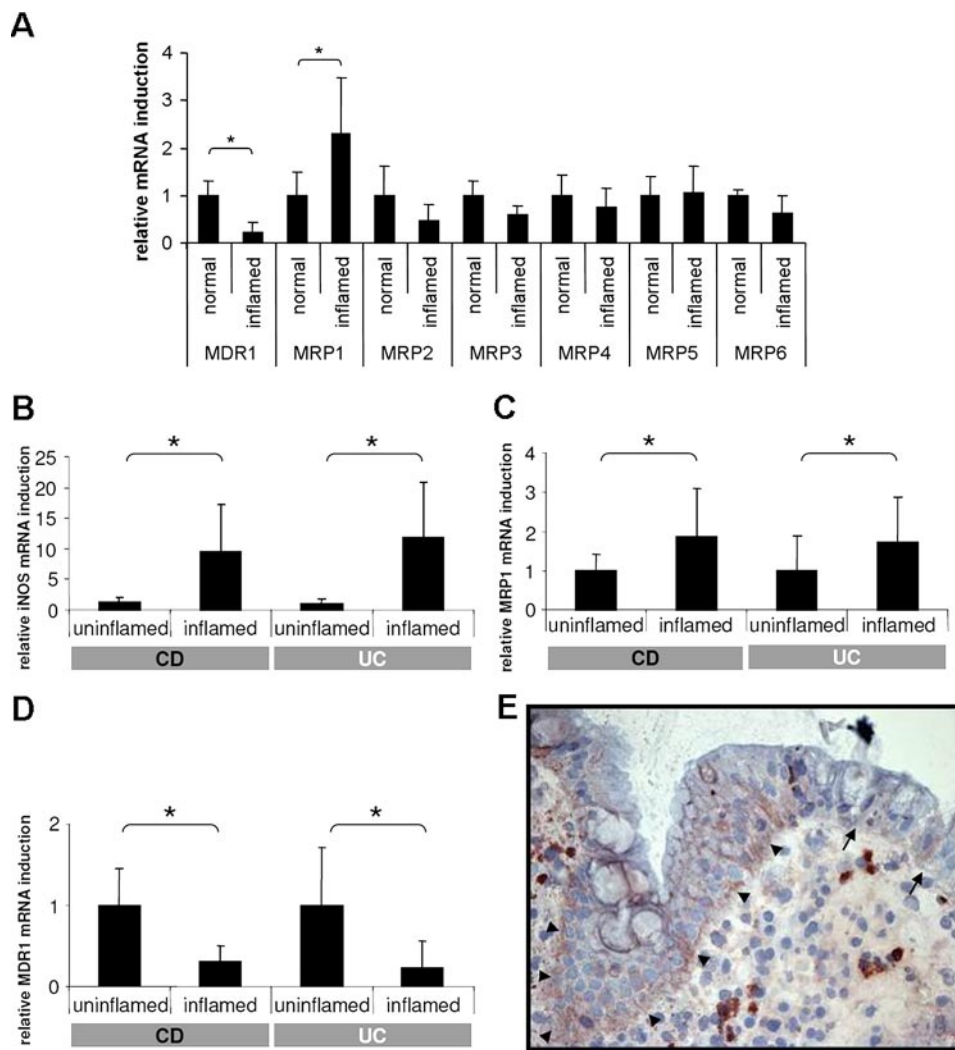


FIGURE 1. Increased MRP1 mRNA and protein expression in inflamed intestinal tissue of CD and UC patients. A, mRNA levels of MDR1 and MRP1–6 in uninflamed (normal) and inflamed tissue of five IBD patients (CD $n = 2$; UC $n = 3$) were determined by real time RT-PCR. Only for MRP1 was a significant increase in inflamed intestinal tissue detected. B–D, mRNA levels of inducible nitric-oxide synthase (B), MRP1 (C), and MDR1 (D) in uninflamed and inflamed tissue of 20 CD and 15 UC patients. MRP1 levels are increased in inflamed intestinal tissue of both patient groups. mRNA levels from uninflamed tissue was set to 1, and relative mRNA expression levels were normalized to 18 S. Data are expressed as means $\pm$ S.D. *, $p < 0.05$ compared with noninflamed samples. E, immunohistochemical localization of MRP1 expression in inflamed colonic tissue of a patient with ulcerative colitis. MRP1-dependent staining is indicated by arrowheads. The original magnification is $\times 200$.

was significantly aggravated in $Mrp1^{-/-}$ knock-out mice exposed to dextran sulfate sodium-induced colitis (19). These animal studies suggest that $Mrp1$ serves a dual role during inflammation, both sending out inflammatory signals as well as protecting the intestinal epithelium. The mechanism of the latter and the relevance for IBD patients are unknown to date.

In this study, we show that MRP1 expression is increased in the inflamed epithelium of patients with IBD, in particular in the intestinal crypts. We demonstrate that MRP1 protects intestinal epithelial cells against cytokine-induced cell death by exporting pro-apoptotic compounds from the cysteinyl leukotriene biosynthesis pathway.

EXPERIMENTAL PROCEDURES

Patient Characteristics—Intestinal mucosal biopsy specimens were obtained during endoscopy following informed con-

sent from patients with IBD. Diagnosis of IBD was established by endoscopic and histopathological examination. The Ethics Committee of the University Hospital Groningen approved the protocol (METc 2002/177c). Intestinal biopsies were obtained from macroscopically inflamed and noninflamed mucosa from 35 patients with IBD using a standard biopsy forceps. Intestinal specimens were immediately snap-frozen in liquid nitrogen for mRNA and protein analysis or liquid nitrogen-cooled isopentane for immunohistochemical staining and stored at -80°C until further processing.

Immunohistochemistry—Immunohistochemistry was performed with the mouse monoclonal antibody QCRL-3 against MRP1 on frozen sections according to the protocol described (dilution 1:10, Santa Cruz Biotechnology, Heidelberg, Germany) (20). The sections were counterstained with hematoxylin. Negative controls consisted of omission of the primary antibody and were consistently negative.

Cell Culture—The human colon carcinoma cell line DLD-1 was cultured as described previously (21). DLD-1 cells were treated with anti-Fas (Immunotech, Marseille, France) or human tumor necrosis factor- α (TNF- α) (R & D Systems Europe, Abingdon, UK). The LTD₄ receptor antagonist MK571 (3-((3-(2-[7-chloro-2-quinolinyl]ethenyl)-phenyl)-((3-(dimethylamino-3-oxopropyl)-thio)-methyl)thio)propanoic acid; Alexis Biochemicals, Lausen,

Switzerland) was used to inhibit MRP function. The 5-lipoxygenase inhibitor AA861 (2-(12-hydroxydodeca-5,10-diynyl)-3,5,6-trimethyl-*p*-benzoquinone; Sigma) was used to inhibit leukotriene synthesis from arachidonic acid.

siRNA-mediated Reduction of MRP1 Expression—DLD-1 cells were seeded in 6-well plates at a density of 400,000 cells per well in RPMI 1640 medium with glutamax supplemented with 1% fetal bovine serum. After 4 h, medium was replaced by serum-free medium. DLD1 cells were transiently transfected with siRNA-MRP1 duplexes using Oligofectamine (Invitrogen) according to the manufacturer's instructions. siRNA-MRP1 primers were obtained from Invitrogen (sense 5'-GGA GUG GAA CCC CUC UCU GdTdT-3' and antisense 5'-CAG AGA GGG GUU CCA CUC CdTdT-3'; kindly provided by Dr. D. J. de Groot, University Medical Center Groningen, Groningen, The Netherlands). Control cells were treated with only Oligo-

fectamine. Four hours after transfection, medium was supplemented with RPMI 1640 medium with 30% fetal bovine serum to adjust the serum concentration to 10%. Subsequently, the cells were cultured in an incubator at 37 °C and 5% CO₂ for 48 h. MRP1 RNA and protein expression was then analyzed by real time PCR and Western blot analysis.

MRP1 Overexpressing Cell Line—DLD-1 cells were seeded in 6-well plates at 300,000 cells per well. The cells were transfected with plasmid pcDNA3.1(–)-MRP1-GFP (22). Transfection was performed using the Transfectin™ lipid reagent protocol (Bio-Rad). Transfected cells were grown to 70% confluence before adding 0.625 mg/ml geneticin to the medium. After 3 days colonies were selected and expanded in medium containing 0.625 mg/ml geneticin. Two clones were further purified by fluorescence-activated cell sorting based on the GFP signal to obtain homogeneous MRP1-expressing cell lines.

RNA Isolation and Quantitative PCR—Total RNA was isolated as described (23). RNA integrity was confirmed by agarose gel electrophoresis, and RNA concentration was measured using a Ribogreen RNA quantitation kit (Molecular Probes, Leiden, The Netherlands). cDNA synthesis was performed on 2.5 µg of total RNA using random primers in a final volume of 50 µl (reverse transcription system, Promega, Madison, WI).

For real time PCR, 4 µl of 20-fold diluted cDNA was used for every PCR in a final volume of 20 µl, containing 900 nmol/liter sense and antisense primers, 200 nmol/liter fluorogenic probe, 5 mmol/liter MgCl₂, 0.2 mmol/liter deoxynucleoside triphosphate mix, 2 µl of real time PCR buffer (10×), and 0.5 units of Hot Goldstar DNA polymerase (Eurogentec, Seraing, Belgium). Details about primers and probes are described in supplemental Table S1. Real time PCR was performed with an ABI Prism 7700 sequence detector version 1.6 software (PerkinElmer Life Sciences). The expression of genes was normalized to the endogenous control (18 S).

Western Blot—Total cell lysates of DLD-1 cells were separated by 10% SDS-PAGE and analyzed by Western blotting. Protein concentrations were determined using the DC protein assay system (Bio-Rad) using bovine serum albumin as standard. Rabbit polyclonal antibody against human PARP (dilution 1:1000; Cell Signaling Technology, Beverly, MA), mouse monoclonal antibody glyceraldehyde-3-phosphate dehydrogenase (dilution 1:5000; Calbiochem), and rat monoclonal antibody MRPr1 were used (dilution 1:500) (24). Horseradish peroxidase-labeled swine anti-rabbit and rabbit anti-mouse IgG were used as secondary antibodies (dilutions 1:2500; DAKO, Heverlee, Belgium).

The blots were exposed in a ChemiDoc XRS system (Bio-Rad). The intensity of the protein bands were quantified using Quantity One software.

Caspase-3 Assay—Caspase-3 enzyme activity was determined according to the manufacturer's instructions using a caspase-3 activity kit with fluorimetric detection (Promega, Leiden, The Netherlands).

Data Analysis—Statistical analyses were performed using SPSS version 12.0 for Windows (SPSS Inc., Chicago). Data obtained from the different groups are expressed as mean values ± S.D. Different groups were compared using Mann-Whit-

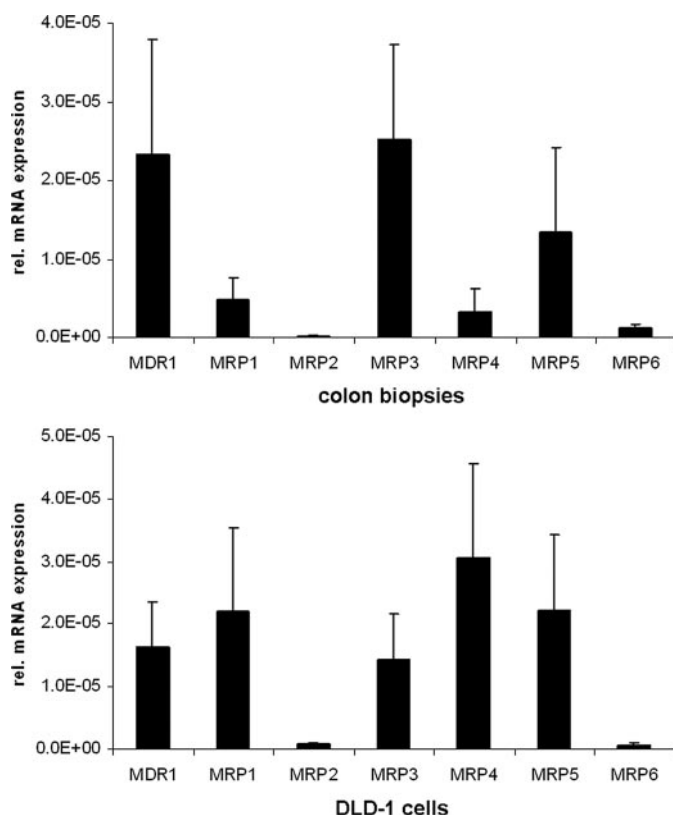


FIGURE 2. MDR1 and MRP1–6 mRNA expression in human colon biopsies and DLD-1 cells. Relative mRNA levels of indicated genes were determined by real time RT-PCR and are corrected for 18 S expression. Data are expressed as means ± S.D.

ney U-tests and Kruskal-Wallis tests. A *p* value of <0.05 was considered as statistically significant.

RESULTS

Expression of MRP1 in Inflamed and Uninflamed Intestinal Tissue—To study the effect of inflammation on ABC transporter expression in the intestinal epithelium, we analyzed bordering mucosal biopsies from uninflamed and inflamed tissue from patients with IBD. In an initial screening, expression of MRP1–6 and MDR1 was analyzed in biopsies from five IBD patients (three with UC and two with CD). MRP1 mRNA levels were significantly increased in inflamed intestinal mucosa, whereas MDR1 expression was down-regulated (Fig. 1A), as described earlier (11). The mRNA levels of MRP2–6 were similar in uninflamed and inflamed intestinal tissue of IBD patients (Fig. 1A). Subsequently, biopsies from 15 UC patients and 20 CD patients were analyzed. Inducible nitric-oxide synthase expression was determined as a measure of inflammation in the biopsies (Fig. 1B). Also in this larger cohort of IBD patients, MRP1 expression was significantly increased in inflamed intestinal mucosa in both UC and CD (Fig. 1C), and MDR1 expression was decreased in both IBD subgroups (Fig. 1D). Immunohistochemistry for MRP1 on colonic biopsy material with high grade inflammation showed a clear basolateral staining of intestinal epithelial cells as well as MRP1-positive mononuclear cells. Notably, MRP1 staining intensity was homogeneous in the epithelial cells lining the whole crypt, whereas the surface epithelium showed minimal MRP1-specific staining (Fig. 1E).

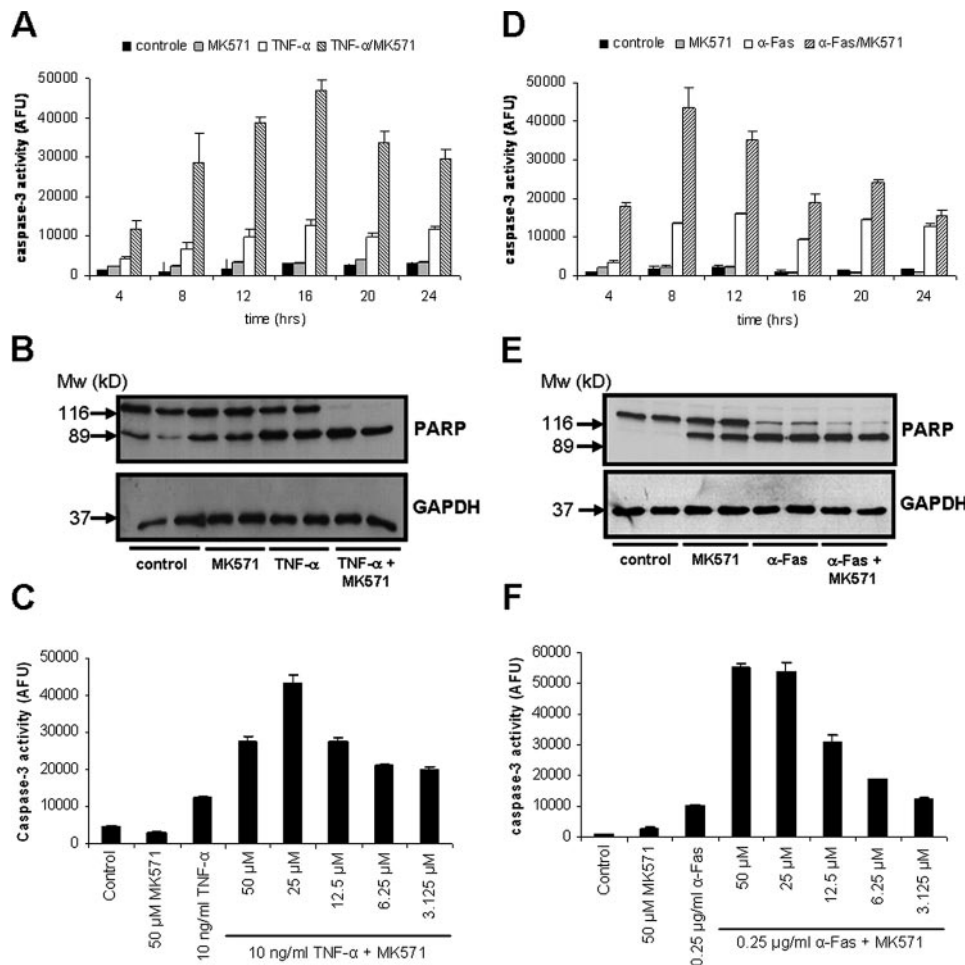


FIGURE 3. Effect of MK571 on anti-Fas- and TNF- α -induced apoptosis of DLD-1 cells. A and D, caspase-3 activity in DLD-1 cells treated for 4–24 h with 10 ng/ml TNF- α (A) or 0.25 μ g/ml anti-Fas (D) in the absence or presence of 50 μ M MK571. B and E, cleavage (activation) of PARP in DLD-1 cells treated for 16 h with TNF- α (B) or 8 h with anti-Fas (E) with and without MK571 (C and F). Duplicate experiments for each condition are shown. Caspase-3 activity in DLD-1 cells treated with TNF- α (C) or anti-Fas (F) in combination with different concentrations of MK571. Data are expressed as means $\pm$ S.D. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. *, $p < 0.05$.

In parallel experiments, the uninflamed tissue from these patients remained unstained for MRP1 (data not shown).

Effect of MK571-mediated Inhibition of MRPs on Anti-Fas- and TNF- α -induced Apoptosis—Increased MRP1 levels may aid cell protection during gastrointestinal inflammatory diseases. Cell damage/death may be induced through receptor-mediated apoptosis in response to FasL or TNF- α , as shown previously in the intestinal mucosa of IBD patients (25–27). To study this putative function, we performed *in vitro* experiments using the human colon epithelial cell line DLD-1. Both human colon biopsies and DLD-1 cells show significant expression of MDR1 and various MRPs, including MRP1 (Fig. 2). This makes DLD-1 cells a useful model for MRP1 inhibition and overexpression studies. First, we tested whether chemical inhibition of MRP-function by MK571 affected DLD-1 cell survival during exposure to anti-Fas or TNF- α . As reported by others (28–31), anti-Fas and TNF- α induce apoptosis in DLD-1 cells in a time-dependent manner with caspase-3 activities peaking at 16 and 12 h for TNF- α and anti-Fas, respectively (Fig. 3, A and D). Concurrently, significant amounts of activated (cleaved) PARP were detected after 16 h of TNF- α (Fig. 3B) or 8 h of anti-Fas

(Fig. 3E) treatment. Adding MK571 strongly increased caspase-3 activities in both TNF- α - and anti-Fas-treated cells (Fig. 3, A and D), which was accompanied by a further increase in the amount of cleaved PARP (Fig. 3, B and E). The increase of TNF- α - or anti-Fas-induced apoptosis was dependent on the concentration of MK571 causing a significant increase of TNF- α - and anti-Fas-induced apoptosis at concentrations as low as 6.25 μ M (Fig. 3, C and F). MK571 (up to 50 μ M tested) itself did not induce caspase-3 activity in DLD-1 cells. Taken together, these results show that inhibition of MRP function strongly sensitizes DLD-1 cells for TNF- α - or anti-Fas-induced apoptosis.

Inhibition of MRP1 Expression by RNA Interference—MK571 is a non-specific MRP inhibitor, and the different MRPs expressed in DLD-1 cells (Fig. 2B) may contribute to a variable extent of cytoprotection. To study the function of MRP1 in this process, we selectively repressed its expression by RNA interference. DLD-1 cells were transiently transfected with MRP1-specific siRNA duplexes, and after 48 h, MRP1 mRNA (Fig. 4A) and protein (Fig. 4, B and C) levels were significantly reduced to $\sim$ 40% of control cells. Exposure of the siRNA-

MRP1-treated DLD-1 cells to anti-Fas led to significantly increased caspase-3 levels (2.0-fold; Fig. 4D). These data show that specific inhibition of MRP1 sensitizes DLD-1 cells to anti-Fas-induced apoptosis and that other MRPs expressed in DLD-1 cells are not able to fully compensate for the loss of MRP1 function.

Stable Overexpression of MRP1 in DLD-1 Cells—Next, we generated DLD-1 cell lines that overexpress MRP1 to examine whether this would increase the resistance of these cells against anti-Fas- and TNF- α -induced apoptosis. DLD-1-derivative cell lines, DLD-MRP1-GFP clone 1 and DLD-MRP1-GFP clone 2, stably overexpress MRP1 tagged at its C terminus to the green fluorescent protein (MRP1-GFP). Previously, it has been shown that this MRP1-GFP hybrid protein retains its substrate transport activity (32). DLD-MRP1-GFP¹ and DLD-MRP1-GFP² contained 17- and 35-fold increased MRP1 mRNA levels, respectively (Fig. 5A). Western blot analysis revealed a strong MRP1-specific signal at $\sim$ 220 kDa, the expected size of the hybrid protein consisting of MRP1 (190 kDa) and GFP (30 kDa) (Fig. 5B). In normal DLD-1 cells, only the endogenous MRP1 protein of $\sim$ 190 kDa was detected. Fluorescence microscopic

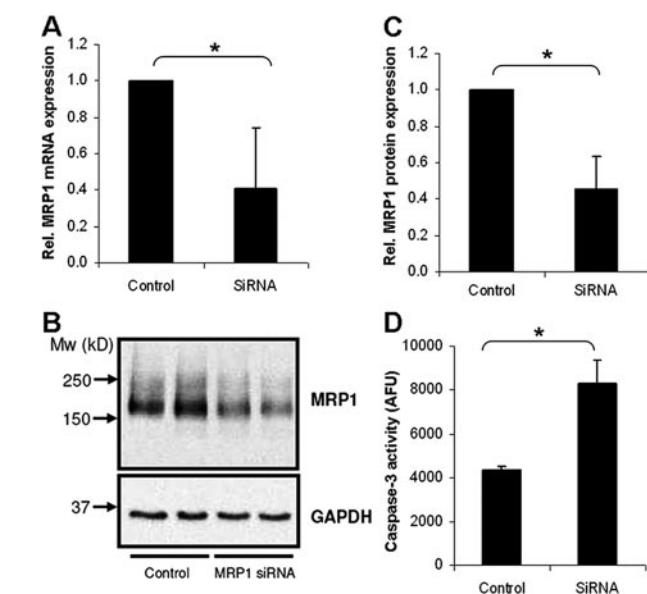


FIGURE 4. RNA interference-induced reduction of MRP1 expression and its effect on anti-Fas-induced apoptosis of DLD-1 cells. MRP1 mRNA (A) and protein (B and C) levels in DLD-1 cells transfected with siRNA duplexes specific for MRP1. Fluorescent non-sense siRNA duplexes were used as a control. A representative Western blot is shown (B). Duplicate experiments for each condition are shown. C shows the quantification of MRP1 Western blot signals from three independent experiments. D, caspase-3 activity in Block-it siRNA (Control) and siRNA-MRP1-treated DLD-1 cells exposed to anti-Fas. Data are expressed as means $\pm$ S.D. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. *, $p < 0.05$.

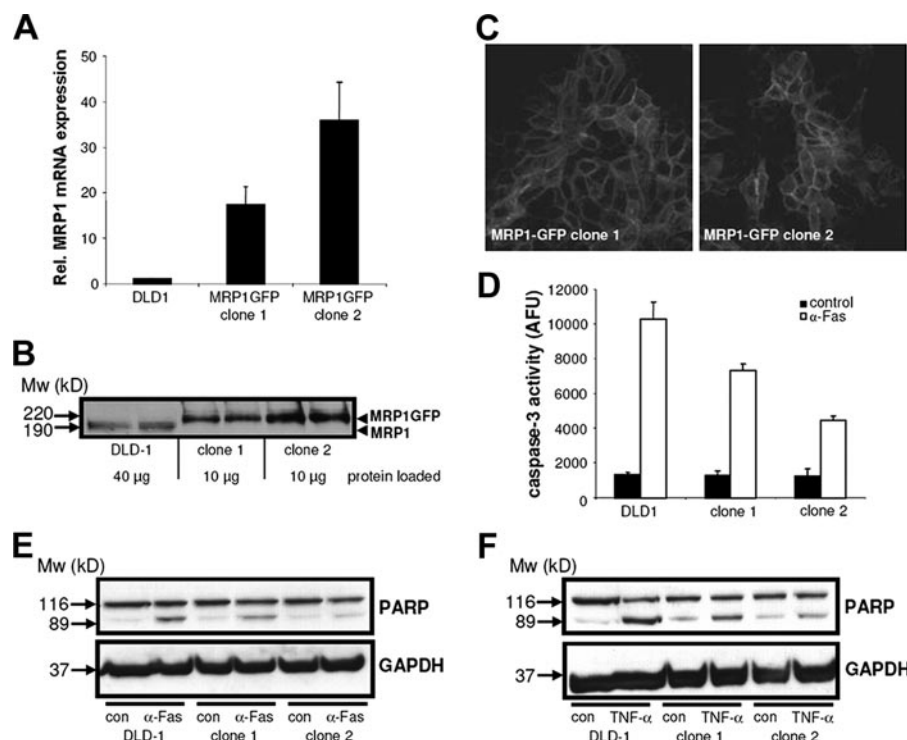


FIGURE 5. Effect of stable overexpression of MRP1 on anti-Fas- or TNF- α -induced apoptosis. MRP1 mRNA (A) and protein (B) expression in DLD-1 cells and two DLD-1-derived cell lines stably transfected with a plasmid producing MRP1-GFP. Western blot analysis revealed the presence of a protein of 220 kDa in both MRP1-GFP-transfected DLD-1-clones, which was detected with the MRP1-specific antibody and had the expected size of GFP-tagged MRP1. Endogenous MRP1 in DLD-1 cells was only detected when at least 40 μ g of total protein extract was analyzed. C, duplicate experiments for each condition are shown. The recombinant MRP1-GFP protein was predominantly present on the plasma membrane (DLD1-MRP1-GFP¹, left panel; DLD1-MRP1-GFP², right panel). Caspase-3 activity (D) and PARP-cleavage (E and F) in DLD-1 cells and the two DLD-1-MRP1-GFP clones exposed for 8 or 16 h to 1 μ g/ml anti-Fas and 10 ng/ml TNF- α , respectively. Western blot detection of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as protein loading control (con).

analysis to determine the subcellular location of the GFP signal revealed a predominant plasma membrane staining in the DLD-MRP1-GFP clones (Fig. 5C), which was not detected in the native DLD-1 cells (data not shown). Exposure of the MRP1 overproducing cell lines to anti-Fas or TNF- α resulted in significantly lower caspase-3 activities and PARP cleavage compared with the native DLD-1 cells (Fig. 5, D–F). Notably, we observed an inverse correlation between the MRP1-GFP expression level and the level of anti-Fas- or TNF- α -induced apoptosis in these cells. These data imply that MRP1 is able to protect cells from anti-Fas- or TNF- α -induced apoptosis.

Inhibition of Cysteinyl Leukotriene Biosynthesis—Inflammation induces the conversion of arachidonic acid into cysteinyl leukotrienes. LTC₄ is a high affinity substrate for MRP1 that is converted to the anti-apoptotic LTD₄ after cellular export. We studied whether blocking MRP function results in the accumulation of a pro-apoptotic compound or, alternatively, prevents the extracellular formation of an anti-apoptotic compound. The lipoxygenase inhibitor AA861 was used to prevent leukotriene synthesis from arachidonic acid. AA861 alone does not induce caspase-3 activity in DLD-1 cells (Fig. 6A). Co-treatment of DLD-1 cells with anti-Fas and increasing amounts of AA861, however, results in a dose-dependent increase in caspase-3 activity. This is probably due to the accumulation of pro-apoptotic arachidonic acid (33, 34). Crucial is the observation that the caspase-3 activity in anti-Fas/AA861-treated cells is significantly lower than that in anti-Fas/MK571-treated cells (Fig. 6A). When DLD-1 cells were then co-treated with anti-Fas, MK571, and increasing amounts of AA861, a dose-dependent decrease in caspase-3 activity was observed (Fig. 6A). At the highest concentration of AA861 (20 μ M), caspase-3 activity in the anti-Fas/MK571/AA861-treated cells was reduced to levels comparable with anti-Fas/AA861-treated DLD-1 cells (Fig. 6A). These data imply that blocking leukotriene synthesis by AA861 results in the accumulation of a pro-apoptotic compound (arachidonic acid) in anti-Fas-treated DLD-1 cells. However, AA861 prevents the intracellular accumulation of an even more potent pro-apoptotic compound in anti-Fas/MK571-treated DLD-1 cells, which is a substrate for MRP1 (Fig. 6B shows a schematic representation of LTC₄ biosynthesis and export and the position and effect of the inhibitors used in this study).

DISCUSSION

In this study, we show that the ABC transporter MRP1 is induced in inflamed intestinal epithelial

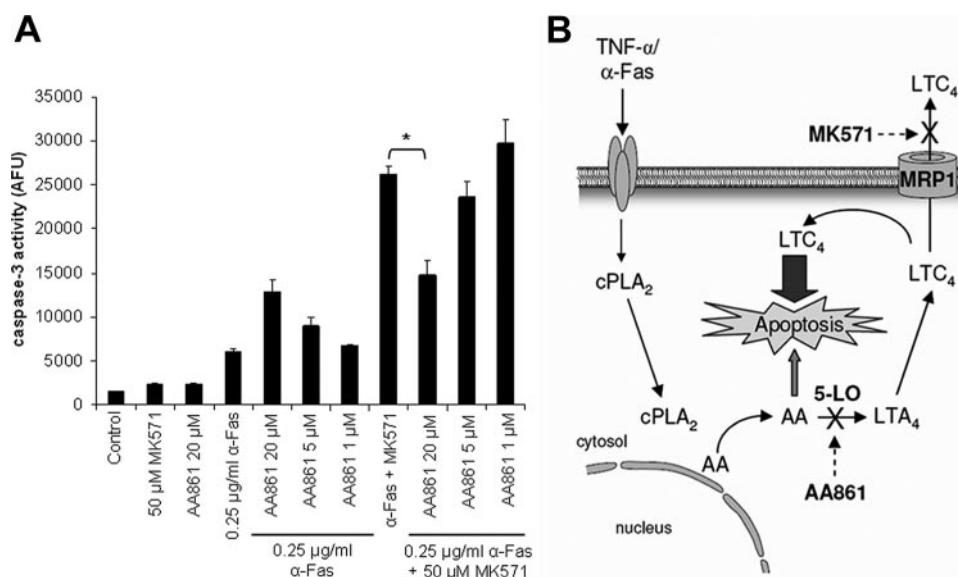


FIGURE 6. Inhibition of leukotriene synthesis in MK-571-treated DLD-1 cells that are exposed to anti-Fas or TNF- α -induced apoptosis. A, caspase-3 activity in DLD-1 cells exposed for 8 h to 0.25 μ g/ml anti-Fas and co-treated with 50 μ M MK571 (MRP1 inhibitor) and/or increasing amounts (1, 5, or 20 μ M) of AA861 (5-lipoxygenase inhibitor). Data are expressed as means $\pm$ S.D. * $p < 0.05$. B, schematic representation of the relationship between MRP1, the leukotriene synthesis pathway, and apoptosis. An inflammatory stimulus leads to receptor-mediated intracellular influx of calcium ions. As a consequence, cytosolic phospholipase A₂ (cPLA₂) translocates from the cytosol to the nuclear membrane. Cytosolic phospholipase A₂ cleaves arachidonic acid (AA) from the membrane phospholipids. Then arachidonic acid is converted by 5-lipoxygenase to leukotriene A₄ (LTA₄), which is converted to LTC₄ through the sequential action of LTC₄ synthase and LTC₄ synthase. LTC₄ is exported by MRP1. Inhibition of 5-lipoxygenase leads to the accumulation of pro-apoptotic arachidonic acid, but the inhibition of MRP1 leads to the accumulation of an even more potent pro-apoptotic agent, LTC₄ (B).

cells of patients with IBD. Pharmacological inhibition of MRP function increased anti-Fas- and TNF- α -induced apoptosis of DLD-1 cells. Specific reduction of MRP1 expression by RNA interference sensitizes DLD-1 cells for anti-Fas-induced apoptosis. Anti-Fas- and TNF- α -induced apoptosis was reduced in DLD-1 cells stably overexpressing MRP1 or when the intracellular accumulation of its high affinity substrate, LTC₄, was prevented by inhibition of 5-lipoxygenase.

MRP1 is best known for its ability to protect tumor cells by stimulating an efflux of drugs that will otherwise induce apoptosis or necrosis of these cells (35). However, MRP1 is also expressed in normal (nonmalignant) tissues such as the colon where it is specifically present in crypt cells that form the proliferative cell compartment of the gut (32, 36, 37). It has been suggested that this may protect these cells against damage induced by xenobiotics (38). We observed that during intestinal inflammation, MRP1 levels were significantly induced. Using immunohistochemistry, we detected a clear basolateral staining for MRP1 of the crypt cells of the inflamed intestine. The increase in MRP1 expression was not part of a general induction of cytoprotective efflux systems, because the expression of another ABC transporter MDR1 was decreased under these conditions (11). Therefore, MRP1 may have a specific role in protecting crypt intestinal epithelial cells during inflammation. We provide four lines of evidence for such a function of MRP1. First, pharmacological inhibition (by MK571) of MRPs strongly increases cytokine- (TNF- α) and anti-Fas-induced apoptosis in DLD-1 cells. Many earlier studies reported that cytokines or anti-Fas induce apoptosis in intestinal and other cell types/lines (28, 29, 39, 40), but this is the first time that a protective role for

anti-Fas-induced apoptosis of T lymphocyte cell lines (Jurkat and MOLT-4) and DLD-1.⁴ We show that cellular glutathione levels are only marginally affected under our experimental conditions and that glutathione depletion is unlikely to be the primary inducing condition for apoptosis in Jurkat and DLD-1 cells.

Second, specific repression of MRP1 expression (by RNA interference) sensitizes DLD-1 cells for anti-Fas-induced apoptosis. The increased level of apoptosis when expression of specifically MRP1 is reduced shows that, of the MRPs present in DLD-1 cells, MRP1 plays the predominant role in cytoprotection against anti-Fas-induced apoptosis. Third, and in line with the previous observation, recombinant overexpression of MRP1 in DLD-1 cells dose-dependently reduced apoptosis induced by anti-Fas or TNF- α . These data suggest that these compounds induce the intracellular production of an apoptotic compound for which MRP1 is the most important efflux pump. TNF- α and anti-Fas are known to induce apoptosis by activation of membrane receptors (TNF-receptor, Fas) (45). Once activated, these receptors recruit adaptor proteins that initiate a signaling pathway, which ultimately leads to activation of effector caspases that mediate apoptosis. No obvious substrate is present in this signaling pathway that may be exported by MRP1, thereby preventing apoptosis. However, TNF- α or anti-Fas may also induce apoptosis through the release of arachidonic acid from the membrane after activation of Ca²⁺-dependent cytosolic phospholipases (46). Arachidonic acid

⁴ A. van Steenpaal, G. Dijkstra, M. Geuken, T. Blokzijl, L. I. H. B. K., H. Moshage, M. Peppelenbosch, and K. N. Faber, submitted for publication.

stimulates apoptosis through activation of sphingomyelinase and through its downstream metabolites, prostaglandins and leukotrienes. Gastrointestinal epithelial cells have been shown to be able to produce these compounds (47). Indeed and as the fourth line of evidence, we found that blocking the biosynthesis of leukotrienes leads to a reduction in TNF- α - or anti-Fas-induced apoptosis in DLD-1 cells with an inhibited MRP function. Because LTC₄ is a high affinity substrate of MRP1, we suggest that this is the pro-apoptotic compound during inflammatory conditions (15). MRP4 and MRP6 have also been shown to be able to transport LTC₄ with relatively high affinity (48, 49), and both transporters are expressed in human intestinal tissue and DLD-1 cells. However, even a partial reduction of MRP1 in DLD-1 cells already leads to sensitization of these cells for anti-Fas-induced apoptosis. Apparently, MRP4 and/or MRP6 cannot fully compensate for the loss of MRP1 expression.

Collectively, these data show that MRP1 has an important role in protecting the intestinal epithelium during inflammation. Our data correlate well with observations by Nishikawa *et al.* (50) who observed that inhibition of LTC₄ synthesis is associated with decreased intestinal damage induced by trinitrobenzene sulfonic acid-induced colitis. In addition, MRP1^{-/-} mice have been shown to develop more severe colitis after administration of dextran sulfate sodium (19, 50). A cytoprotective role of MRP1 against inflammation-induced cell death may not be restricted to intestinal epithelial cells. Previously, we found that in livers of patients with severe hepatitis, hepatic progenitor cells contain high levels of MRP1 (51). Similar to the intestinal crypt cells, hepatic progenitor cells are involved in regeneration of damaged tissue during pathophysiological conditions. They need to survive in conditions when they are exposed to cytokines, toxic metabolites, and xenobiotics. MRP1 may be an important component of cellular cytoprotection during inflammation.

In conclusion, our data show a novel function of MRP1, which is protection of intestinal epithelial cells against inflammation-induced apoptosis. MRP1 expression is increased in intestinal epithelial cells, in particular those lining the crypt, which may serve to preserve the intestinal regenerative capacity.

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**Up-regulation and Cytoprotective Role of Epithelial Multidrug
Resistance-associated Protein 1 in Inflammatory Bowel Disease**

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