Decreased P-glycoprotein (P-gp/MDR1) Expression in Inflamed Human Intestinal Epithelium is Independent of PXR Protein Levels

Hans Blokzijl, MD, Sara Vander Borght,† Lisette I.H. Bok,* Louis Libbrecht, MD, PhD,† Mariska Geuken,* Fiona A.J. van den Heuvel, MD,* Gerard Dijkstra, MD, PhD,* Tania A.D. Roskams, MD, PhD,† Han Moshage, PhD,* Peter L.M. Jansen, MD, PhD,‡ and Klaas Nico Faber, PhD**

Background: Altered P-glycoprotein expression (P-gp/MDR1) and/or function may contribute to the pathogenesis of gastrointestinal inflammatory disorders. Low intestinal mRNA levels of the pregnane X receptor (PXR) have been linked to low MDR1 mRNA levels in patients with ulcerative colitis (UC). Here we compared intestinal MDR1 mRNA and protein expression in uninflamed and inflamed intestinal epithelium (IE) of patients with gastrointestinal inflammatory disorders to healthy controls.

Methods: Intestinal mucosal biopsies were obtained from patients with Crohn's disease (CD, $n = 20$), UC ($n = 10$), diverticulitis ($n = 3$), collagenous colitis ($n = 3$), and healthy controls ($n = 10$). MDR1, iNOS, MRP1, CYP3A4, and PXR expression was determined using real-time reverse-transcriptase polymerase chain reaction (RT-PCR), Western blotting, and/or immunohistochemistry. Furthermore, MDR1 expression was determined in human intestinal biopsies and the human colon carcinoma cell line DLD-1 after exposure to cytokines (TNF-α, IFN-γ, and/or IL-1β).

Results: MDR1 mRNA levels in uninflamed colon of UC patients were comparable to healthy controls, while they were slightly decreased in ileum and slightly increased in colon of CD patients. MDR1 expression, however, was strongly decreased in inflamed IE of CD, UC, collagenous colitis, and diverticulitis patients. A cytokine-dependent decrease of MDR1 expression was observed in human intestinal biopsies, but not in DLD-1 cells. Remarkably, PXR protein levels were equal in uninflamed and inflamed tissue of CD and UC patients despite low PXR mRNA levels in inflamed tissue.

Conclusions: MDR1 expression is strongly decreased in inflamed IE of patients with gastrointestinal disorders and this is independent of PXR protein levels. Low MDR1 levels may aggravate intestinal inflammation.

(*Inflamm Bowel Dis 2007;13:000–000)*

Key Words: P-glycoprotein/multidrug resistance 1, intestinal epithelium, inflammation, pregnane X receptor

A single layer of intestinal epithelial cells (IECs) make up the barrier between the host and the luminal content of the intestine. The cells of the mucosal immune system are protected against the luminal antigen load by this single layer of epithelial cells. Disturbance of the integrity of the epithelial cell barrier contributes to the development of mucosal inflammation.[1] Many proteins are expressed by epithelial cells that are directly or indirectly involved in maintaining the protective barrier, including substrate transport proteins, tight junction proteins, and trefoil peptides.[2–4] An additional important function of the epithelium is the facilitation of transepithelial transport of essential nutrients and the efflux of potentially toxic compounds. Many of these transport functions are performed by proteins belonging to the ATP-binding cassette (ABC) transporter family.[5]

P-glycoprotein (P-gp/MDR1), encoded by the *ABCB1* gene, is an 170-kDa transmembrane protein that is highly expressed at the apical side of the intestinal epithelium (IE).[6] Its function is to mediate efflux of compounds from the mucosa to the gut lumen. There is increasing evidence that changes in MDR1 function and/or expression contribute to the pathogenesis of inflammatory disorders of the gastrointestinal tract.[7] Inflammatory bowel disease (IBD)-susceptibility loci were found on chromosome 7, in which the *MDR1* gene is located.[8] Indeed, single nucleotide polymorphisms

Received for publication November 16, 2006; accepted November 20, 2006.

This article includes Supplementary Material available via the Internet at http://www.interscience.wiley.com/jpages/1078-0998/suppmat.

From the *Department of Gastroenterology and Hepatology, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands; †Department of Morphology and Molecular Pathology, University of Leuven, Belgium; ‡Department of Gastroenterology and Hepatology, Academic Medical Center, Amsterdam, The Netherlands.

Funded by a grant from the Dutch Digestive Diseases Foundation (MLDS WS 01-22), the J.K. de Cock Foundation, and AstraZeneca. L.L. is a postdoctoral researcher of the FWO-Vlaanderen. K.N.F. is supported by a VIDI fellowship from the Netherlands Organization for Scientific Research (NWO).

Reprints: Klaas Nico Faber, PhD, Department of Gastroenterology and Hepatology, University Medical Center Groningen, PO-Box 30.001, 9700 RB Groningen, The Netherlands (e-mail: k.n.faber@med.umcg.nl).

DOI 10.1002/ibd.20088

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

TABLE 1. Baseline Characteristics

Characteristics	Crohn's Disease	Ulcerative Colitis	Collagenous Colitis	Diverticulitis	Healthy Controls
Mean age, yr (range)	40 (20–62)	48 (27–78)	69 (63–73)	61 (54–68)	38 (17–56)
Sex (M/F)	4/16	4/6	0/3	1/2	6/4
Mean duration of disease					
de novo, no. patients	10/20	0/10	—	—	—
long-term disease, no. patients	10/20	10/10	—	—	—
yr (range)	10 (3–39)	9 (1–23)	—	—	—
Concomitant medication, (yes/no)	8/12	8/2	1/2	1/2	0/10
Corticosteroids	6	4	1	1	—
Sulfasalazine/mesalazine	2	7	—	—	—
Azathioprine	3	2	—	1	—
Methotrexate	1	—	1	—	—

Results are expressed as means and ranges.

(SNPs) in the human *MDR1* gene have recently been reported that show association with IBD.[9–11] Also, the low mRNA levels of the pregnane X receptor (PXR) and its target gene MDR1 in uninflamed intestinal tissue of ulcerative colitis (UC), as recently reported by Langmann et al,[12] are in line with the association of MDR1 with IBD. However, not all genetic studies support the association of specific MDR1 SNPs and IBD. For instance, no association of the C3435T polymorphism with UC or Crohn's disease (CD) was detected in large cohorts of UC and CD patients from northern Europe and Greece, respectively.[13,14]

Animal studies do support a possible association of Mdr1 function and intestinal inflammation. Panwala et al[2] showed that Mdr1a$^{-/-}$ mice developed spontaneous intestinal inflammation,[15,16] which is further aggravated when these animals are exposed to *Helicobacter bilis*. These experiments support the belief that a compromised IE barrier is involved in the development of IBD.[17]

Besides a primary effect of SNPs on MDR1 function, it is also important to determine the effect of inflammation on MDR1 expression. Contradicting data have been reported about hepatic Mdr1 regulation during inflammation. In LPS-treated rats we showed that Mdr1b in the liver is strongly increased in an NF-κB-dependent manner, whereas Mdr1a expression is unchanged.[18,19] Other authors showed that hepatic Mdr1a and Mdr1b are downregulated in endotoxemic rats and mice in an IL-1- and IL-6-dependent manner.[20–23] Recent data suggest that inflammation also results in decreased levels of intestinal Mdr1 in rats[24] and mice,[25] but human MDR1 expression is increased in Caco-2 cells treated with proinflammatory cytokines.[26]

No data are available yet on the effect of inflammation on the expression of MDR1 in CD or UC, let alone what kind of regulatory mechanisms may be involved. The aim of this project was to study the intestinal expression of human MDR1 and to determine the role of the transcriptional regulator of MDR1 PXR in the inflamed epithelium.

MATERIALS AND METHODS

Patient Characteristics

Intestinal mucosal biopsy specimens were obtained during endoscopy from patients with CD or UC and control patients. Intestinal material from patients with diverticulitis and collagenous colitis was used for the disease control group. Diagnosis of IBD, diverticulitis, and collagenous colitis was established by endoscopic, radiologic, and histopathologic examination. The controls were patients referred to our endoscopy center because of abdominal cramp and diarrhea with unknown cause, Irritable Bowel Syndrome (IBS), polyp surveillance, obstipation, changed stool frequency, and anemia of unknown cause. The control patients had no mucosal inflammation upon endoscopic and histologic examination. Patient characteristics are described in Table 1. The Ethics Committee of the University Medical Center Groningen approved the protocol (METc 2002/177c).

Tissue Specimens

Intestinal biopsies were obtained from macroscopically inflamed and noninflamed mucosa from the colon in UC patients and from the terminal ileum and the colon in CD patients. In addition, biopsies were taken from 4 different intestinal areas (ileum, ascending colon, transverse colon, and rectum) in 10 control patients using a standard biopsy forceps. As disease controls, we used intestinal tissue that was obtained after sigmoid resection of 3 patients with diverticulitis and intestinal biopsies of 3 patients with collagenous colitis. Intestinal specimens were immediately snap-frozen in liquid nitrogen for mRNA and protein analysis or in dry ice-cooled isopentane for immunohistochemical staining and

TABLE 2. Sequences of Primers and Probes used for Real-time Detection PCR Analysis

18S	sense	5′-CGG CTA CCA CAT CCA AGG A-3′
	antisense	5′-CCA ATT ACA GGG CCT CGA AA-3′
	probe	5′FAM-CGC GCA AAT TAC CCA CTC CCG A-TAMRA3′
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′FAM-CGT GTC CCA GGA GCC CAT CCT GT-TAMRA3′
iNOS	sense	5′-GGC TCA AAT CTC GGC AGA ATC-3′
	antisense	5′-GGC CAT CCT CAC AGG AGA GTT-3′
	probe	5′FAM-TCC GAC ATC CAG CCG TGC CAC-TAMRA3′
MRP1	sense	5′-CTT CTG GAG GAA TTG GTT GTA TAG AAG-3′
	antisense	5′-GGT AGA CCC AGA CAA GGA TGT TAG A-3′
	probe	5′FAM-TCT TTG AGA TGC TTC TGG CTC CCA TCA C-TAMRA3′
CYP3A4	sense	5′-GCA GGA GGA AAT TGA TGC AGT T-3′
	antisense	5′-GTC AAG ATA CTC CAT CTG TAG CAC AGT-3′
	probe	5′FAM-ACC CAA TAA GGC ACC ACC CAC CTA TGA-TAMRA3′
PXR	sense	5′-AGC AAT TCG CCA TTA CTC TGA AGT-3′
	antisense	5′-CTG GGT GTG CTG AGC ATT GA-3′
	probe	5′FAM-AGA TCA TGG CTA TGC GCA CCG AGC TCC-TAMRA3′

stored at −80°C until further processing. Resection material from patients with diverticulitis and intestinal biopsies from patients with collagenous colitis were embedded in paraffin after fixation in 6% formalin.

Cell Culture Experiments

The human colon carcinoma cell line DLD-1 or fresh biopsy specimens obtained from control patients were used for in vitro experiments. Biopsies were taken from macroscopic and microscopic normal mucosa from the transverse colon. Human biopsies and DLD-1 cells were cultured in a humidified incubator at 37°C in 5% CO_2 in RPMI medium (Invitrogen, Breda, The Netherlands) supplemented with glutamax, 10% v/v fetal calf serum (FCS, Gibco BRL, Breda, The Netherlands), penicillin (50 U/mL), streptomycin (50 μg/mL), and fungizone (5 μg/mL) (BioWhittaker, Verviers, Belgium). DLD-1 cells and human colon biopsies were incubated for 8 hours with a cytokine mix (CM) composed of 10 ng/mL human tumor necrosis factor-α (TNF-α, R&D Systems Europe, Abingdon, UK), 10 ng/mL human interleukin-1β (IL-1β, R&D Systems Europe), and 10 ng/mL human interferon-γ (IFN-γ, R&D Systems Europe). Prior to the CM-treatment, DLD-1 cells were placed on fresh serum-free culture medium.

RNA Isolation and Quantitative Polymerase Chain Reaction (PCR)

Total RNA was isolated as described previously.[27] RNA was checked on an agarose gel for integrity and RNA concentration was measured using Ribogreen RNA quantitation kit (Molecular Probes, Leiden, The Netherlands). Reverse transcription was performed on 2.5 mg of total RNA using random primers in a final volume of 50 μL (Reverse Transcription System, Promega, Madison, WI). Samples were heated for 10 minutes at 25°C, followed by 60 minutes at 37°C, and subsequently for 5 minutes at 94°C to terminate the reverse transcription reaction.

For real-time PCR, 4 μL 20-fold diluted complementary DNA was used for every PCR reaction in a final volume of 20 μL, containing 900 nmol/L sense and antisense primers, 200 nmol/L fluorogenic probe, 5 mmol/L $MgCl_2$, 0.2 mmol/L deoxynucleoside triphosphate mix, 2 μL real-time PCR buffer (10×), and 0.5 U Hot Goldstar DNA Polymerase (Eurogentec, Seraing, Belgium). Details of primers and probes are described in Table 2. Fluorescence was measured by an ABI Prism 770 Sequence Detector version 1.6 software (Perkin Elmer Life Sciences, Foster City, CA) initialized by 10 minutes at 95°C to denature the complementary DNA followed by 40 PCR cycles each of 95°C for 15 seconds and 60°C for 1 minute. Each sample was measured in duplicate. Standard curves for both the gene of interest and the endogenous control were obtained. A standard curve was derived from serial dilution of a pool of cDNA from the samples analyzed. Subsequently, the cycle threshold values were transformed using the standard curve into relative amounts. Finally, the gene of interest was normalized with respect to our endogenous control (18S).

Immunohistochemistry

Immunohistochemistry was performed on intestinal frozen and paraffin-embedded sections using the primary monoclonal antibodies JSB-1 against MDR1 (dilution 1/10;

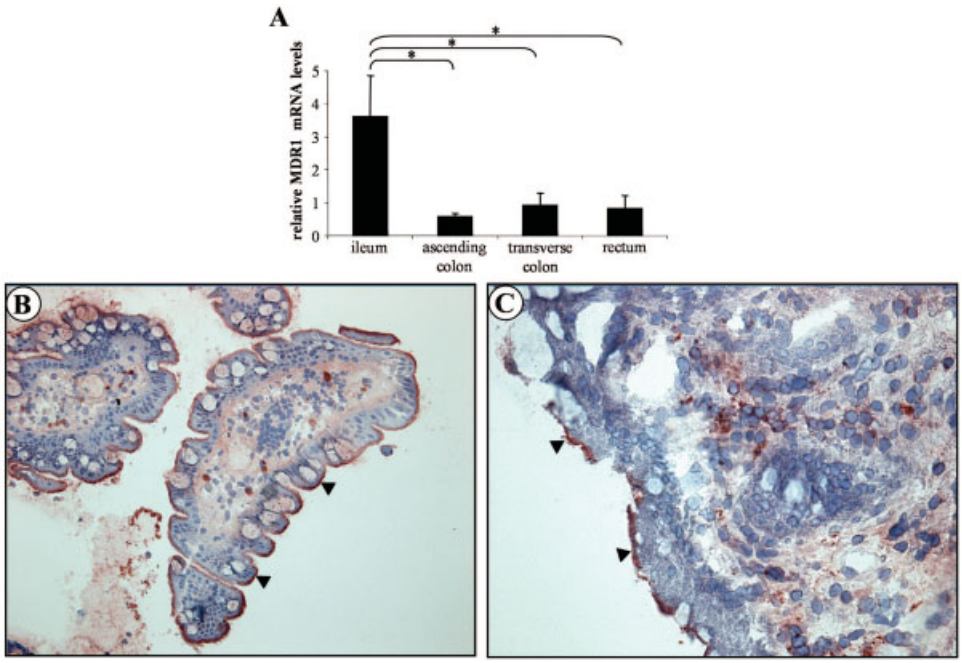


FIGURE 1. MDR1 expression throughout the human colon and in the ileum. Biopsies were taken from control patients ($n = 10$) and analyzed for MDR1 mRNA levels by real-time RT-PCR (A) and cell-specific MDR1 protein expression using immunohistochemistry in ileum (B) and colon (C). MDR1 mRNA levels were equal throughout the colon and ≈5-fold higher in the ileum ($P < 0.05$ compared to ileum). Typical MDR1-specific staining (using the JSB1 antibody) was observed at the apical membrane of ileal and colon epithelial cells. Epithelial staining was continuous in the ileum and "patchy" in the colon.

Monosan, Uden, The Netherlands) and KL1 against cytokeratin (dilution 1:50, Immunotech, Marseille, France).

Staining of frozen sections for MDR1 and KL1 was performed according to the protocol described previously.[28] MDR1 staining was performed on 5-μm-thick paraffin-embedded sections, which were deparaffinized in xylene followed by rehydration. Endogenous peroxidase activity was blocked using 0.3% H_2O_2 in methanol for 20 minutes. Before staining, antigen retrieval was performed in 10 mM citrate buffer (pH 6.0). The sections were incubated at room temperature with primary antibodies for 30 minutes. Subsequently, the slides stained for MDR1 were incubated for 30 minutes at room temperature with rabbit antimouse biotinylated IgG (dilution 1:400; Dako, Carpinteria, CA) and then incubated with avidin biotin complex/horseradish peroxidase (HRP). All incubation steps were followed by a wash in 3 changes of phosphate-buffered saline (PBS). The reaction product was developed with the use of 3-amino-9-ethylcarbazol (DAB). For negative controls the primary antibody was omitted.

To check the viability of the in vitro human intestinal biopsies after culturing, we performed hematoxylin and eosin staining. At 0 and 8 hours biopsies were imbedded in paraffin, cut in 4-μm-thick sections, and subsequently stained.

Western Blot

Total cell lysates of human biopsies or transfected DLD-1 cells were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by Western blotting. Protein concentrations were determined using the Bio-Rad DC Protein Assay system (Bio-Rad, Munich, Germany) using bovine serum albumin as standard. The rabbit polyclonal antibody against human PXR (dilution 1:750, Active Motif, Rixensart, Belgium) and monoclonal antibodies anti-iNOS/NOS Type II (dilution 1:300, Becton Dickinson, Alphen a/d Rijn, The Netherlands), C219 against P-glycoprotein (dilution 1:1000; Signet Laboratories, Dedham, MA), and GAPDH were used (dilution 1:5000, Calbiochem, Darmstadt, Germany). HRP-labeled swine antirabbit IgG and rabbit antimouse IgG were used as secondary antibodies (dilutions 1:2500; Dako). Detection was performed using Chemiluminescent Substrate Luminol/Enhancer (Cell Signaling Technology, Beverly, MA). DLD-1 cells transfected with plasmid CDG1-hSXR (kindly provided by B. Blumberg) served as positive control for human PXR detection by Western blotting.[29]

Statistics

Statistical analyses were performed using SPSS v. 12.0 for Windows (SPSS, Chicago, IL). Data obtained from the different groups are expressed as mean values ± standard deviation (SD). Different groups were compared using Mann–Whitney U tests and Kruskal-Wallis tests. A P value of < 0.05 was considered statistically significant.

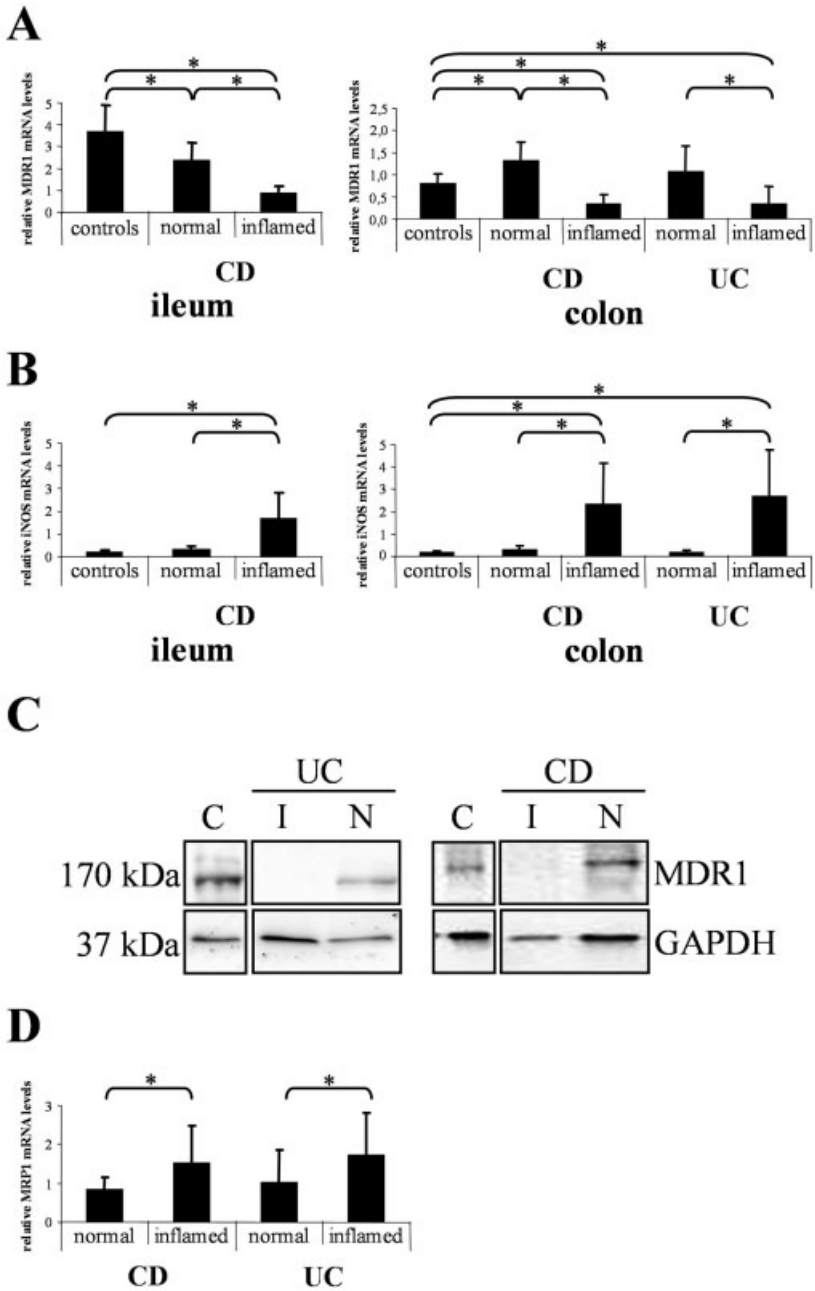


FIGURE 2. MDR1 mRNA expression is decreased in inflamed intestinal tissue from IBD patients. Biopsies were taken from 1) uninflamed (normal) and inflamed tissue of CD ($n = 20$) and UC ($n = 10$) patients, and 2) normal tissue from control patients ($n = 10$) and subsequently analyzed for mRNA and protein levels of MDR1 (A,C), the inflammation marker iNOS (B), and MRP1 (D) using real-time RT-PCR and Western blotting. MDR1 mRNA levels in noninflamed tissue in CD were significantly increased in the colon and decreased in the ileum compared to control patients; in contrast, no significant difference was observed between noninflamed tissue in UC and healthy controls (A). iNOS mRNA levels were significantly increased in inflamed intestinal biopsies from patients with CD or UC (B). MDR1 mRNA and protein expression was significantly decreased in the inflamed tissue of patients with CD or UC (A,C). In contrast, the expression of MRP1 was significantly increased (D). C, control; N, normal; I, inflamed. *$P < 0.05$.

RESULTS

MDR1 Expression in the IE of Control Patients

Biopsies were taken from the terminal ileum, ascending colon, transverse colon, and the rectum in control patients and were processed for MDR1 mRNA quantification and immunohistochemistry. MDR1 mRNA levels were constant throughout the colon. MDR1 mRNA levels in the terminal ileum were 5-fold higher than in the colon (Fig. 1A). Using immunohistochemistry, a strong apical MDR1 staining of the IE surface was observed. Epithelial staining was con-

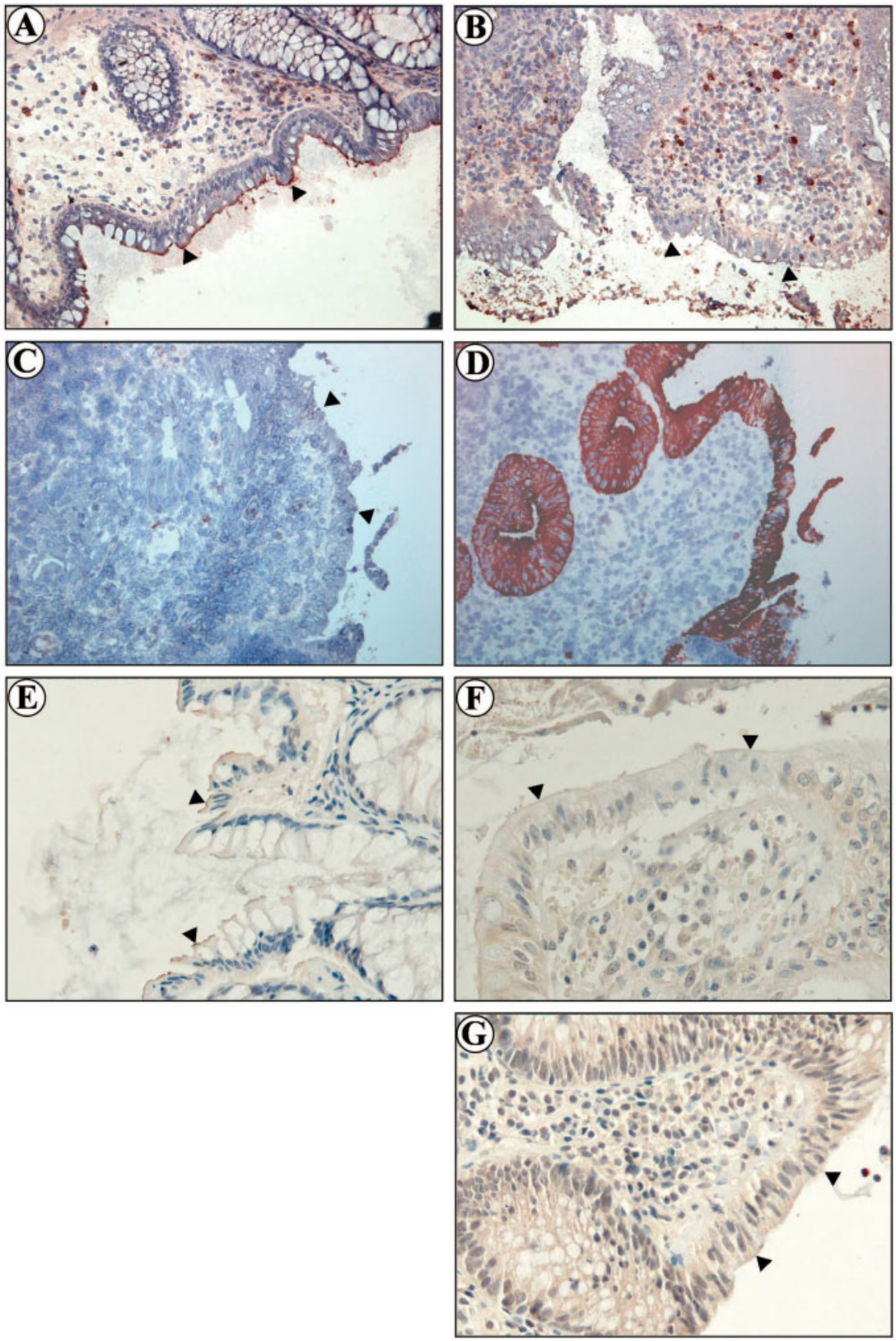


FIGURE 3

tinuous in the ileum (Fig. 1B) and somewhat "patchy" in the colon (Fig. 1C). No differences were observed in MDR1 staining throughout the colon. Besides IECs, scattered mononuclear cells were stained positive for MDR1 in the lamina propria, but mononuclear aggregates were negative for MDR1 (Fig. 1B). MDR1 expression was equal throughout the colon. Thus, effects of inflammation can be compared in biopsies taken from different parts of the colon.

Expression of MDR1 in Uninflamed IE of IBD Patients

Several SNPs in MDR1 have been suggested to be linked to IBD. Such SNPs may give rise to decreased MDR1 mRNA and/or protein levels.[30–34] Therefore, we analyzed the MDR1 mRNA expression in uninflamed tissue of our IBD patient group and compared those to healthy controls. Figure 2A shows that in UC patients the MDR1 mRNA level in the uninflamed colon was not significantly different compared with healthy controls, with a trend of being slightly increased. The MDR1 mRNA levels in the uninflamed colon of CD patients were also increased (+67%) and here a statistical significant difference was observed. Remarkably, in the uninflamed ileum of CD patients a minor, but significantly lower (64% of normal) MDR1 mRNA level was detected (Fig. 2A). Immunohistochemistry for MDR1 showed an apical staining indistinguishable from the one observed in the controls (Fig. 3A, compare with Fig. 1C). These data show that MDR1 levels in uninflamed IE may slightly differ from that observed in healthy controls, but that the specific effect within 1 patient group may be different for the ileal and the colon epithelium.

Decreased MDR1 Expression in Inflamed IE of IBD Patients

Next we determined the effect of inflammation on the expression of MDR1. Biopsies were taken from uninflamed and inflamed tissue in IBD patients within a distance of a few centimeters. Inducible NOS (iNOS) mRNA expression was determined to confirm active inflammation in the biopsy. iNOS mRNA levels in uninflamed tissue of IBD patients were comparable to controls, but strongly increased (>10-fold) in inflamed ileum and colon tissue of these patients (Fig. 2B). In contrast to iNOS, MDR1 mRNA and protein levels were strongly decreased in inflamed tissue from CD as well as from UC patients (Fig. 2A,C). The decreased MDR1 mRNA level resulted in a complete disappearance of MDR1-dependent immunohistochemical staining in inflamed IECs (Fig. 3B). Importantly, staining for cytokeratin showed that the epithelium was still intact (Fig. 3C,D). The absence of epithelial MDR1-staining was accompanied with a significant increase in the number of MDR1-positive mononuclear cells (Fig. 3B). This implies that the decrease in MDR1 mRNA in the inflamed IECs is likely underestimated. We did not observe any difference in MDR1 mRNA levels between patients with or without medication (Fig. S1). The decrease of MDR1 was not part of an overall downregulation of (ABC)-transporters in inflamed intestinal tissue, since the expression of the multidrug resistance-associated protein 1 (MRP1) was increased in this patient group (Fig. 2D).

Decreased Intestinal MDR1 Expression in Inflamed Mucosa is not Specific for IBD

To investigate whether the observed decrease of MDR1 protein was specific for inflamed tissue of IBD patients, immunohistochemistry for MDR1 was performed on intestinal biopsies from patients with collagenous colitis and on resection material from patients with diverticulitis. Similar to the results obtained for IBD patients, MDR1-staining was absent in the inflamed intestinal tissue of patients with diverticulitis or collagenous colitis (Fig. 3F,G). Again, uninflamed IE stained positive for MDR1 (Fig. 3E).

Cytokine-dependent Expression of MDR1 in Human Intestinal Biopsies and the Intestinal Cell Line DLD-1

In vitro experiments are required to study the molecular mechanism(s) involved in downregulation of intestinal MDR1. Others have shown that cytokines induce MDR1 mRNA expression in Caco-2 cells.[26] We obtained similar results when analyzing cytokine-dependent regulation of MDR1 in the human colon carcinoma cell line DLD-1. None of the individual cytokines tested was able to induce any significant reduction of MDR1 mRNA levels (Fig. 4A). Consequently, these cell lines cannot be used to delineate the mechanism involved in intestinal MDR1 downregulation. Therefore, we performed similar experiments with freshly isolated biopsies from the transverse colon from control patients. An 8-hour incubation of these biopsies in the presence of a cytokine mixture consisting of TNF-α, IL-1β, and INF-γ resulted in a significant reduction of the

FIGURE 3. MDR1 protein levels are strongly decreased in inflamed intestinal epithelium. Biopsies were taken from uninflamed (A,E) and inflamed (B–D,F,G) tissue from the colon of patients with UC (A–D), diverticulitis (E,F), or collagenous colitis (G) and processed for immunohistochemistry using the MDR-1-specific antibody JSB-1 (A–C,E–G) or the cytokeratin specific antibody KL1 (D). A strong apical MDR1-staining was observed of the epithelium of the noninflamed mucosa of UC patients (A), which was absent in the inflamed tissue (B). Staining of serial sections of inflamed tissue for either MDR1 (C) or cytokeratin (D) shows that the intestinal epithelium is still intact. Apical MDR1 staining is also observed in noninflamed tissue of patients with diverticulitis (E), which is absent in inflamed tissue of the same patient (F). Apical MDR1 is also undetectable in inflamed tissue of a patient with collagenous colitis (G).

MDR1 mRNA level in combination with a 7-fold increase of the iNOS mRNA level (Fig. 4C,D). Importantly, the histological architecture remained intact during the 8 hours in vitro incubation, without any significant signs of increased necrotic or apoptotic cells (Fig. 4E). These results correlate well with the in vivo observations described above. The individual cytokines all gave rise to reduced MDR1 expression without, however, reaching statistical relevance. Clearly, the combination of the different cytokines, which is a better reflection of the in vivo situation during inflammation, resulted in the strongest, and statistically significant, MDR1 mRNA decrease.

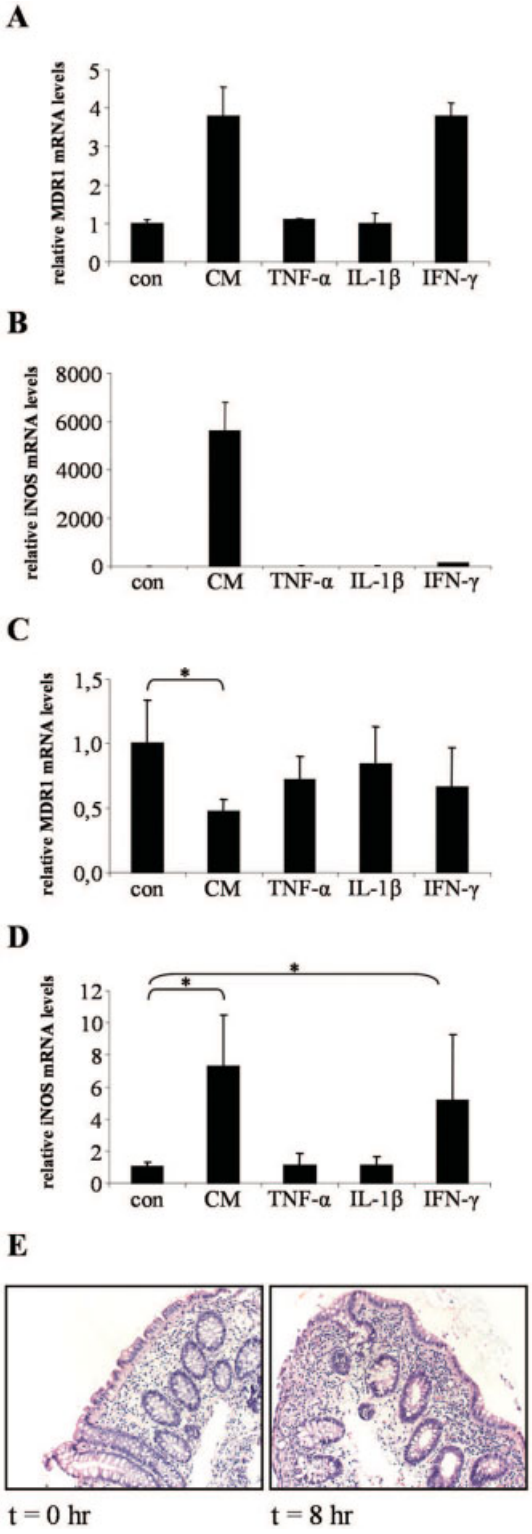


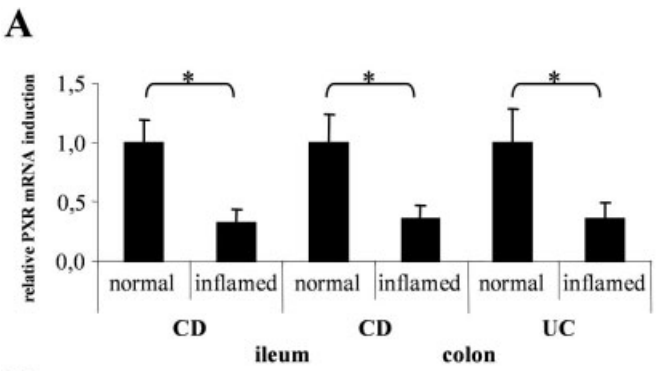
FIGURE 4. Cytokine-induced decrease in MDR1 mRNA expression in human colon biopsies. DLD-1 (A,B) or human biopsies from the transverse colon of control patients (C,D) were incubated for 8 hours with 10 ng/mL TNF-α, 10 ng/mL IL-1β, 10 ng/mL INF-γ, or a combination of these 3 cytokines (CM). MDR1 expression was increased in DLD-1 cells after incubation with cytokine mix (A). INF-γ administration resulted in the strongest increase of both iNOS and MDR1 levels compared with the other two cytokines (A,B). Treatment of human intestinal biopsies with CM resulted in a significant reduction of MDR1 mRNA levels (C), while iNOS mRNA expression was significantly increased (D). Treatment of human biopsies with the individual cytokines did not result in a significant change in MDR1 mRNA levels. Data represent mean values of biopsies from 5 different patients. The viability of the cultured intestinal biopsies was evaluated by immunohistochemistry. Histological integrity of the biopsies proved to be intact (E). *$P < 0.05$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Downregulation of MDR1 is Independent of PXR Protein Levels

Recently, intestinal downregulation of MDR1 mRNA in patients with UC was suggested to be caused by decreased PXR mRNA levels, a transcriptional regulator of the MDR1 gene.[12] Therefore, we studied the expression of PXR in our tissue samples. Similar to MDR1, we found that PXR mRNA levels were significantly decreased in inflamed intestinal tissue of both groups of IBD patients (Fig. 5A). However, PXR protein levels did not follow the decrease in PXR mRNA levels. The PXR protein level remained remarkably constant even if the PXR mRNA level had dropped to ≈4% in the inflamed versus uninflamed tissue from 1 patient (Fig. 5B). In addition, mRNA expression of the PXR-regulated gene cytochrome P450 3A4 (CYP3A4) was not changed or increased in 7 (out of 30) patients, while MDR1 was downregulated (Fig. 6). Taken together, these results do not support the involvement of PXR in downregulation of intestinal MDR1 during inflammation.

DISCUSSION

Reduced MDR1 function is associated with increased risk of gastrointestinal inflammatory disorders, as shown by genetic linkage studies of patients with IBD and animal experiments.[2,9–11] No data were yet available about the effect of inflammation per se in the intestinal epithelium of patients



B

mRNA levels	Patient 1		Patient 2		Patient 3	
	normal	inflamed	normal	inflamed	normal	inflamed
iNOS	0,36	1,96	0,28	2,06	0,08	0,40
PXR	1,0	0,51	1,0	0,19	1,0	0,04

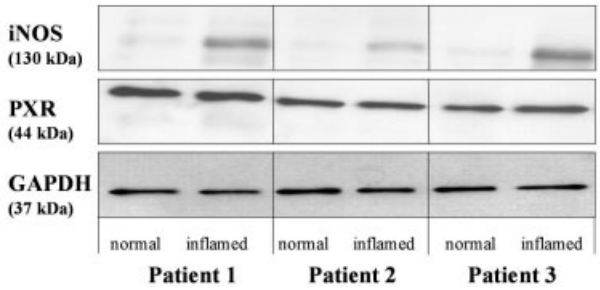


FIGURE 5. Downregulation of MDR1 is independent of PXR protein levels. Biopsies were taken from uninflamed (normal) and inflamed tissue of CD ($n = 20$) and UC ($n = 10$) patients and analyzed for PXR mRNA (A) and protein levels (B). A significant decrease in PXR mRNA levels was observed in the inflamed tissue compared to uninflamed tissue (A; $P < 0.05$). Western blot detection of PXR in total protein extracts of biopsies from uninflamed and inflamed tissue revealed similar levels of PXR protein. Patient 1, UC (rectum); Patient 2, CD (ascending colon); Patient 3, CD (ileum). Specificity of the antibody was shown with PXR-transfected DLD-1 cells (B). Quantification of PXR mRNA levels in biopsies taken from the same sites revealed strongly reduced PXR mRNA levels in the inflamed tissue of these patients (B, table). The inflammation marker iNOS was strongly increased in the inflamed intestinal tissue of these patients, both at the mRNA level and the protein level (B).

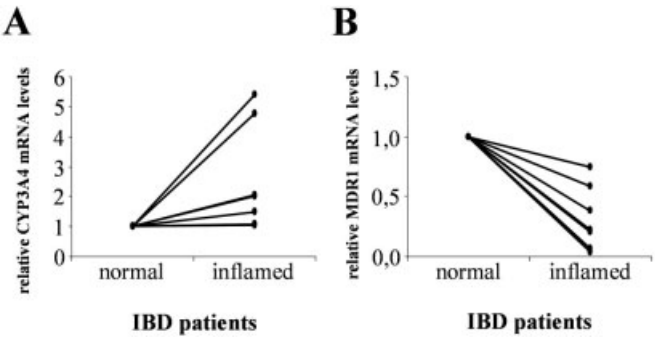


FIGURE 6. The PXR-target genes MDR1 and CYP3A4 are not strictly coregulated in the noninflamed versus inflamed tissue of patients with CD or UC. MDR1 and CYP3A4 mRNA levels were quantified in biopsies from noninflamed and inflamed tissue of CD ($n = 20$) and UC ($n = 10$) patients. In 7 of these patients, CYP3A4 mRNA levels were unchanged or increased (A), while MDR1 mRNA levels were decreased (B).

with IBD. Here we show that MDR1 expression is strongly compromised in the inflamed epithelium of these patients. Cytokine-dependent reduction of MDR1 transcription was shown in in vitro experiments using intestinal biopsies from control patients. Negative regulation of MDR1 was independent of PXR protein levels.

MDR1 is well known for its ability to export a great variety of drugs out of (cancer) cells. In normal tissue it is highly expressed in the intestine, underscoring its potential role in determining drug bioavailability and elimination of naturally occurring toxins in food. Physiological substrates for MDR1 have not yet been firmly established, but MDR1 has been proposed to play a role in migration of antigen-presenting dendritic cells and may be able to transport cytokines like IL-2 and IL-4.[35,36] Clearly, changes in MDR1 function and/or expression may have major implications for proper functioning of the intestine.

Langmann et al[12] recently showed that MDR1 expression was reduced in uninflamed sections of the colon of UC patients, whereas no significant difference was observed in the ileum of UC patients, nor in the colon or ileum of CD patients. In contrast, we found no significant difference in the uninflamed tissue of UC patients, with a trend of being slightly increased. Remarkably, we found a slight but significant increase of MDR1 in uninflamed colon of CD patients, concomitantly with a slight but significant decrease in the uninflamed ileum of these patients. The observed increase in MDR1 levels in noninflamed tissue in the colon could be an adaptive mechanism to compensate for the decreased expression in inflamed tissue. Similar observations have been reported by Buyse et al[25] who found an increased Mdr1 expression and activity in noninflamed ileal tissue in mice with DSS-induced colitis. The most significant effect we observed in our study was the very strong difference in MDR1 expression in inflamed compared to noninflamed intestinal tissue obtained from individual patients. MDR1 expression was strongly decreased in inflamed tissue of patients with CD, UC, diverticulitis, or collagenous colitis. These data are in line with Th1- and Th2-mediated experimental models of colitis, in which a significant decrease of Mdr1a expression was also observed.[37] Our data, however, seem to contradict the observations made by Farrell et al.[38] Using flow cytometry, they found that IECs from uninflamed or inflamed mucosa of UC patients contained similar MDR1 expression

levels. At present, we cannot explain this apparent contradiction, rather than that it may be related to the methods used. Our analyses on biopsies were performed on immediately fixed or snap-frozen material, thereby minimizing the effects of in vitro incubations after obtaining the biopsies. It is unknown whether the isolation procedure for IECs as performed by Farrell et al may affect initial differences in MDR1 expression in uninflamed versus inflamed tissues.

No cytokine-dependent downregulation of MDR1 has been established using intestine-derived cell lines, such as Caco-2.[12,26] Also, the DLD-1 cell line used in this study showed a strong increase in MDR1 transcription upon incubation with a cytokine-mixture consisting of TNF-α, IL-1β, and INF-γ. However, we were able to establish a cytokine-dependent downregulation of MDR1 using freshly isolated intestinal biopsies from healthy controls. All individual cytokines gave rise to a slight decrease in MDR1 expression without reaching statistical significance. A significant 50% reduction in MDR1 expression was observed when the 3 cytokines were used simultaneously. Clearly, the transcriptional regulation of MDR1 in colon carcinoma cell lines differs from what is observed in intestinal cells in vivo. In vitro incubation of freshly isolated intestinal biopsies provides a good experimental model to study this.

Transcriptional regulation of the MDR1 gene is complex. Many different transcription factors have been shown to bind to the DNA in the MDR1 promoter region and directly control its transcription. The nuclear receptor PXR is 1 of these transcription factors, which binds to a region 8 kb upstream of the MDR1 transcription start site and positively regulates MDR1 transcription.[39] PXR is a ligand-activated transcription factor and a key regulator of the xenobiotic response system that coordinates the expression of genes involved in metabolism and transport of toxic compounds. SNPs in the *NR1I2* gene encoding PXR have been identified that affect PXR activity and thereby the expression of target genes including MDR1, CYP3A4, and several UDP-glucuronosyltransferase isoforms.[40–42] More recently, SNPs in PXR have been shown to be associated with the development of IBD.[43] In addition, low PXR mRNA levels in patients with UC have recently been linked to low MDR1 mRNA levels in uninflamed IE of these patients.[12] Although we did not observe reduced levels of PXR in the healthy IE of IBD patients versus controls, we did find that PXR mRNA levels were significantly decreased in inflamed tissue when compared to uninflamed tissue. Remarkably, however, PXR protein levels were comparable in uninflamed and inflamed tissue from individual patients, even if the PXR mRNA level was reduced 25-fold. This finding again shows that mRNA levels may strongly diverge from the level of the corresponding protein and, in our case, unlink the relationship between PXR and MDR1 mRNA levels. Still, one needs to bear in mind that PXR is a ligand-activated transcription factor and its activation state in uninflamed and inflamed tissue has not been studied to date. However, we also observed that another PXR target gene, CYP3A4, may be upregulated in inflamed tissue of IBD patients that simultaneously showed a downregulation of MDR1. This further underscores our view that low MDR1 levels are not a result of changed PXR levels and/or activation.

What may be the physiological consequences of reduced MDR1 function in the intestine? MDR1 is primarily known for its capacity to export drugs out of cells. In IECs it directs its substrates to the gut lumen.[5] Obviously, reduced levels may therefore increase the bioavailability of drugs used to treat these patients, such as glucocorticoids and immunosuppressants.[44] However, due to the local inflammation of the intestinal mucosa in most IBD patients, the influence on drug availability is most probably limited. On the other hand, the MDR1-dependent protection against naturally occurring toxins is compromised in the inflamed intestine. This is also substantiated by the fact that intestinal bacteria are required to initiate and/or aggravate the inflammation in the mdr1$^{-/-}$ knockout mouse.[2] MDR1 has also been implicated in the transport of cytokines[36] and chemokines,[45,46] as well as protecting IECs against apoptosis[47] and regulating dendritic cell migration.[35,48] Future research needs to resolve which of the above function(s) of MDR1 are aiding the diseased conditions of IBD patients.

ACKNOWLEDGMENT

The authors thank Dr. B. Blumberg for the kind gift of the CDG1-hSXR plasmid.

REFERENCES

1. Podolsky DK. Mucosal immunity and inflammation. V. Innate mechanisms of mucosal defense and repair: the best offense is a good defense. *Am J Physiol.* 1999;277:G495–G499.
2. Panwala CM, Jones JC, Viney JL. A novel model of inflammatory bowel disease: mice deficient for the multiple drug resistance gene, mdr1a, spontaneously develop colitis. *J Immunol.* 1998;161:5733–5744.
3. Mitic LL, Van Itallie CM, Anderson JM. Molecular physiology and pathophysiology of tight junctions I. Tight junction structure and function: lessons from mutant animals and proteins. *Am J Physiol Gastrointest Liver Physiol.* 2000;279:G250–G254.
4. Mashimo H, Wu DC, Podolsky DK, et al. Impaired defense of intestinal mucosa in mice lacking intestinal trefoil factor. *Science.* 1996;274:262–265.
5. Borst P, Elferink RO. Mammalian ABC transporters in health and disease. *Annu Rev Biochem.* 2002;71:537–592.
6. Thiebaut F, Tsuruo T, Hamada H, et al. Cellular localization of the multidrug-resistance gene product P-glycoprotein in normal human tissues. *Proc Natl Acad Sci U S A.* 1987;84:7735–7738.
7. Ho GT, Moodie FM, Satsangi J. Multidrug resistance 1 gene (P-glycoprotein 170): an important determinant in gastrointestinal disease? *Gut.* 2003;52:759–766.
8. Satsangi J, Parkes M, Louis E, et al. Two stage genome-wide search in inflammatory bowel disease provides evidence for susceptibility loci on chromosomes 3, 7 and 12. *Nat Genet.* 1996;14:199–202.
9. Brant SR, Panhuysen CI, Nicolae D, et al. MDR1 Ala893 polymorphism is associated with inflammatory bowel disease. *Am J Hum Genet.* 2003; 73:1282–1292.
10. Schwab M, Schaeffeler E, Marx C, et al. Association between the C3435T MDR1 gene polymorphism and susceptibility for ulcerative colitis. *Gastroenterology.* 2003;124:26–33.

11. Hoffmeyer S, Burk O, von Richter O, et al. Functional polymorphisms of the human multidrug-resistance gene: multiple sequence variations and correlation of one allele with P-glycoprotein expression and activity in vivo. *Proc Natl Acad Sci U S A.* 2000;97:3473–3478.
12. Langmann T, Moehle C, Mauerer R, et al. Loss of detoxification in inflammatory bowel disease: dysregulation of pregnane X receptor target genes. *Gastroenterology.* 2004;127:26–40.
13. Croucher PJ, Mascheretti S, Foelsch UR, et al. Lack of association between the C3435T MDR1 gene polymorphism and inflammatory bowel disease in two independent northern European populations. *Gastroenterology.* 2003;125:1919–1920.
14. Gazouli M, Zacharatos P, Gorgoulis V, et al. The C3435T MDR1 gene polymorphism is not associated with susceptibility for ulcerative colitis in Greek population. *Gastroenterology.* 2004;126:367–369.
15. Banner KH, Cattaneo C, Le Net JL, et al. Macroscopic, microscopic and biochemical characterisation of spontaneous colitis in a transgenic mouse, deficient in the multiple drug resistance 1a gene. *Br J Pharmacol.* 2004;143:590–598.
16. Wilk JN, Bilsborough J, Viney JL. The mdr1a-/- mouse model of spontaneous colitis: a relevant and appropriate animal model to study inflammatory bowel disease. *Immunol Res.* 2005;31:151–160.
17. Maggio-Price L, Shows D, Waggie K, et al. Helicobacter bilis infection accelerates and H. hepaticus infection delays the development of colitis in multiple drug resistance-deficient (mdr1a-/-) mice. *Am J Pathol.* 2002;160:739–751.
18. Vos TA, Hooiveld GJ, Koning H, et al. Up-regulation of the multidrug resistance genes, Mrp1 and Mdr1b, and down-regulation of the organic anion transporter, Mrp2, and the bile salt transporter, Spgp, in endotoxemic rat liver. *Hepatology.* 1998;28:1637–1644.
19. Ros JE, Schuetz JD, Geuken M, 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.
20. Piquette-Miller M, Pak A, Kim H, et al. Decreased expression and activity of P-glycoprotein in rat liver during acute inflammation. *Pharm Res.* 1998;15:706–711.
21. Sukhai M, Yong A, Kalitsky J, et al. Inflammation and interleukin-6 mediate reductions in the hepatic expression and transcription of the mdr1a and mdr1b genes. *Mol Cell Biol Res Commun.* 2000;4:248–256.
22. Sukhai M, Yong A, Pak A, et al. Decreased expression of P-glycoprotein in interleukin-1beta and interleukin-6 treated rat hepatocytes. *Inflamm Res.* 2001;50:362–370.
23. Hartmann G, Vassileva V, Piquette-Miller M. Impact of endotoxin-induced changes in P-glycoprotein expression on disposition of doxorubicin in mice. *Drug Metab Dispos.* 2005;33:820–828.
24. Kalitsky-Szirtes J, Shayeqanpour A, Brocks DR, Piquette-Miller M. Suppression of drug-metabolizing enzymes and efflux transporters in the intestine of endotoxin-treated rats. *Drug Metab Dispos.* 2004;32:20–27.
25. Buyse M, Radeva G, Bado A, et al. Intestinal inflammation induces adaptation of P-glycoprotein expression and activity. *Biochem Pharmacol.* 2005;69:1745–1754.
26. Bertilsson PM, Olsson P, Magnusson KE. Cytokines influence mRNA expression of cytochrome P450 3A4 and MDRI in intestinal cells. *J Pharm Sci.* 2001;90:638–646.
27. Plass JR, Mol O, Heegsma J, et al. Farnesoid X receptor and bile salts are involved in transcriptional regulation of the gene encoding the human bile salt export pump. *Hepatology.* 2002;35:589–596.
28. Ros JE, Libbrecht L, Geuken M, et al. 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.
29. Blumberg B, Sabbagh W Jr, Juguilon H, et al. SXR, a novel steroid and xenobiotic-sensing nuclear receptor. *Genes Dev.* 1998;12:3195–3205.
30. Hitzl M, Drescher S, van der Kuip H, et al. The C3435T mutation in the human MDR1 gene is associated with altered efflux of the P-glycoprotein substrate rhodamine 123 from CD56+ natural killer cells. *Pharmacogenetics.* 2001;11:293–298.
31. Hitzl M, Schaeffeler E, Hocher B, et al. Variable expression of P-glycoprotein in the human placenta and its association with mutations of the multidrug resistance 1 gene (MDR1, ABCB1). *Pharmacogenetics.* 2004;14:309–318.
32. Nakamura T, Sakaeda T, Horinouchi M, et al. Effect of the mutation (C3435T) at exon 26 of the MDR1 gene on expression level of MDR1 messenger ribonucleic acid in duodenal enterocytes of healthy Japanese subjects. *Clin Pharmacol Ther.* 2002;71:297–303.
33. Oselin K, Gerloff T, Mrozikiewicz PM, et al. MDR1 polymorphisms G2677T in exon 21 and C3435T in exon 26 fail to affect rhodamine 123 efflux in peripheral blood lymphocytes. *Fundam Clin Pharmacol.* 2003; 17:463–469.
34. Siegmund W, Ludwig K, Giessmann T, et al. The effects of the human MDR1 genotype on the expression of duodenal P-glycoprotein and disposition of the probe drug talinolol. *Clin Pharmacol Ther.* 2002;72:572–583.
35. Randolph GJ, Beaulieu S, Pope M, et al. A physiologic function for p-glycoprotein (MDR-1) during the migration of dendritic cells from skin via afferent lymphatic vessels. *Proc Natl Acad Sci U S A.* 1998;95:6924–6929.
36. Drach J, Gsur A, Hamilton G, et al. Involvement of P-glycoprotein in the transmembrane transport of interleukin-2 (IL-2), IL-4, and interferon-gamma in normal human T lymphocytes. *Blood.* 1996;88:1747–1754.
37. Mizoguchi E, Xavier RJ, Reinecker HC, et al. Colonic epithelial functional phenotype varies with type and phase of experimental colitis. *Gastroenterology.* 2003;125:148–161.
38. Farrell RJ, Murphy A, Long A, et al. High multidrug resistance (P-glycoprotein 170) expression in inflammatory bowel disease patients who fail medical therapy. *Gastroenterology.* 2000;118:279–288.
39. Geick A, Eichelbaum M, Burk O. Nuclear receptor response elements mediate induction of intestinal MDR1 by rifampin. *J Biol Chem.* 2001; 276:14581–14587.
40. Hustert E, Zibat A, Presecan-Siedel E, et al. Natural protein variants of pregnane X receptor with altered transactivation activity toward CYP3A4. *Drug Metab Dispos.* 2001;29:1454–1459.
41. Gardner-Stephen D, Heydel JM, Goyal A, et al. Human PXR variants and their differential effects on the regulation of human UDP-glucuronosyltransferase gene expression. *Drug Metab Dispos.* 2004;32:340–347.
42. Zhang J, Kuehl P, Green ED, et al. The human pregnane X receptor: genomic structure and identification and functional characterization of natural allelic variants. *Pharmacogenetics.* 2001;11:555–572.
43. Dring MM, Goulding CA, Trimble VI, et al. The pregnane X receptor locus is associated with susceptibility to inflammatory bowel disease. *Gastroenterology.* 2006;130:341–348.
44. Farrell RJ, Kelleher D. Glucocorticoid resistance in inflammatory bowel disease. *J Endocrinol.* 2003;178:339–346.
45. Honig SM, Fu S, Mao X, et al. FTY720 stimulates multidrug transporter- and cysteinyl leukotriene-dependent T cell chemotaxis to lymph nodes. *J Clin Invest.* 2003;111:627–637.
46. Raggers RJ, Vogels I, Van Meer G. Multidrug-resistance P-glycoprotein (MDR1) secretes platelet-activating factor. *Biochem J.* 2001;357:859–865.
47. Johnstone RW, Cretney E, Smyth MJ. P-glycoprotein protects leukemia cells against caspase-dependent, but not caspase-independent, cell death. *Blood.* 1999;93:1075–1085.
48. Randolph GJ. Dendritic cell migration to lymph nodes: cytokines, chemokines, and lipid mediators. *Semin Immunol.* 2001;13:267–274.