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Breast Cancer Resistance Protein and P-Glycoprotein Expression in Patients with Newly Diagnosed and Therapy-Refractory Ulcerative Colitis Compared with Healthy Controls

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Key Words

Breast cancer resistance protein · P-glycoprotein · Transporter expression · Ulcerative colitis · Therapy refractoriness · Inflammation

Abstract

Aims: Efflux transporters such as breast cancer resistance protein (BCRP/ABCG2) and P-glycoprotein (Pgp; MDR1/ABCB1) are protecting the enterocytes from potentially toxic compounds. Both transporters have been reported to be downregulated in patients with active ulcerative colitis (UC). The aim of this study was to evaluate transporter expression in both unaffected and inflamed mucosa of patients with active UC, in drug-naïve and treated patients with UC and compare the results with transporter expression in healthy subjects. ***Methods:*** Transporter expression was determined with real-time RT-PCR (TaqMan) in inflamed and unaffected mucosa of newly diagnosed (n = 12) and therapy-refractory (n = 11) patients with UC. Expression levels were compared with UC patients in remission (n = 11) and control subjects (n = 26). BCRP and Pgp expression was evaluated by immunohistochemistry. ***Results:*** Compared with unaffected mucosa, BCRP expression was significantly reduced in inflamed mucosa of newly diagnosed drug-naïve patients with UC (expression reduced to 30%) as well as in patients not responding to treatment (reduced to 25%) with either 5-aminosalicylates (n = 7) or prednisone (n = 4). Unaffected mucosa of UC patients showed comparable transporter expression to unaffected mucosa of control subjects. MDR1 expression depicts a similar pattern. Protein staining for Pgp and BCRP was significantly reduced in the inflamed mucosa of patients with active UC. ***Conclusions:*** Expression of both efflux transporters BCRP and MDR1 is reduced, but only in inflamed tissue of patients with active UC. Transporter expression in unaffected mucosa of patients with active UC is comparable to healthy controls. The data suggest that the inflammatory process is responsible for the reduced levels. A major role in the pathogenesis of UC is unlikely.

H.G. and P.H. contributed equally to this study.

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Introduction

Breast cancer resistance protein (BCRP/ABCG2) as well as P-glycoprotein (Pgp; MDR1/ABCB1) are members of the ATP-binding cassette (ABC) transporter family. Both were first detected in multi-drug resistant tumor cells [1, 2]. Both have a protective function against potentially toxic xenobiotics and are highly expressed in tissues that are important for uptake and elimination of toxic substances such as the intestine, the kidney, the liver, and the blood brain barrier [3–7]. BCRP and Pgp are localized in the apical membrane of intestinal cells, where they limit the absorption of orally administered drugs and ingested toxins such as the BCRP substrates PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine), benzo[a]pyrene [8] and the chlorophyll-derived phototoxin pheophorbide a [9, 10] or the Pgp substrates digoxin, ciclosporin or verapamil [11–13]. These findings indicate that both transporters exhibit a role in maintaining the barrier function of the intestinal epithelium. Several drugs (such as dexamethasone [14] and sulphasalazine [15]) used in the treatment of inflammatory bowel disease are transported by Pgp and BCRP.

Previous studies with gene arrays and quantitative RT-PCR have reported that MDR1/ABCB1 (the gene of Pgp), and BCRP are downregulated in patients with active ulcerative colitis (UC) [16–19]. Genetic variation analysis of MDR1 indicates that this gene locus is associated with susceptibility for UC [20, 21].

These studies did, however, not differentiate between the transporter expression patterns in tissue samples obtained from inflamed or unaffected mucosa of the same individuals and did not take into account whether the patients with UC received previous or concomitant anti-inflammatory treatment. Since it is well known that several drugs such as the antimycobacterial drug rifampicin or the antidepressant St. John's wort are able to change the expression of Pgp [22, 23], it is important to investigate whether the medication used in UC might influence MDR1 and/or BCRP mRNA expression.

Therefore, MDR1 and BCRP expression was investigated in newly diagnosed untreated patients with UC, patients with UC not responding to anti-inflammatory medication, UC patients in remission and healthy controls. In patients with active UC, it was of special interest to evaluate transporter expression in unaffected and inflamed mucosa. We hypothesized that gene expression of MDR1 and BCRP may be altered in UC.

Materials and Methods

Patient Characteristics

In this study, 12 patients with newly diagnosed UC, 11 patients with active UC (refractory to treatment), 11 patients in remission and 26 control subjects were enrolled after having given their informed consent. For detailed patient characteristics, see table 1. Diagnosis of patients with UC was based on a typical clinical history, laboratory findings as well as endoscopic and histological criteria. Tissue biopsies were sampled by experienced gastroenterologists. From patients with active disease (newly diagnosed patients and patients with refractory disease) biopsies were taken from the inflamed and from the unaffected region. In two newly diagnosed patients, biopsies could only be obtained of the inflamed mucosa due to macroscopic signs of pancolitis. Unaffected areas were defined as mucosa regions without any macroscopic/endoscopic signs of inflammation (ulceration, edema, discoloration, hemorrhagic appearance or mucinous/fibrinous coating); these biopsies were obtained at least 10–15 cm distant from the pathologic area. Control subjects had an indication for a gastrointestinal tract endoscopy within a cancer screening program. Biopsy specimens were obtained during routine endoscopy from the colon, submerged in RNA-later solution (Qiagen, Hilden, Germany) and stored at –80°C until further processing. The study protocol and consent forms were approved by the State Ethical Committee of Basel prior to the start of the study.

Real-Time RT-PCR Analysis (TaqMan)

Total RNA was isolated from 2 biopsies from each subject. RNA was extracted using the RNeasy Mini Kit (Qiagen) following the instructions provided by the manufacturer. RNA was quantified with a GeneQuant photometer (Pharmacia, Uppsala, Sweden). After DNase I digestion (Gibco Life Technologies, Basel, Switzerland), 1.5 μg of total RNA was reverse-transcribed by Superscript (Gibco Life Technologies) according to the manufacturer's protocol using random hexamers as primers.

TaqMan analysis was carried out on a 7900HT Sequence Detection System (Applied Biosystems, Rotkreuz, Switzerland) as previously described in recent papers of our group [4, 24, 25]. In brief, standards for mRNA quantification were obtained by classical PCR using duodenal cDNA as a template. Primers for generating the standards and primers/probes for TaqMan analysis were designed according to the guidelines of Applied Biosystems with the help of the Primer Express 2.0 software (corresponding sequences are displayed in table 1). All samples were run in triplicates and were quantified using a standard curve. Not reverse-transcribed RNA served as a negative control. For each sample the transcript numbers of BCRP, MDR1 and of villin were determined. By calculating the ratio of BCRP/villin and MDR1/villin, the mRNA expression was normalized. This approach has been established to account for variations in the enterocyte content of biopsies [3, 26].

Immunohistochemistry

Human duodenal tissue fragments were mounted in OCT compound (Sakura Finetek, Zooterwoude, The Netherlands), snap-frozen in liquid nitrogen and stored at –80°C. Frozen sections (5 μm) were air dried overnight and a periodate-lysine paraformaldehyde solution (3%) was used for postfixation. Then

Table 1. Patient characteristics

	Patients newly diagnosed with UC (n = 12)	Patients with active UC refractory to therapy (n = 11)	Patients in remission (n = 11)	Control subjects (n = 26)
Median age (range), years	55 (27–78)	55 (27–77)	42 (24–69)	56 (24–78)
Sex	4 M/8 F	7 M/4 F	4 M/7 F	13 M/13 F
Mean BMI ± SEM	23.9 ± 0.9	24.5 ± 1.5	24.3 ± 0.9	27.8 ± 1.2
Mean clinical activity index ± SEM	5.2 ± 0.7	6.8 ± 0.9	1.4 ± 0.5	–
Medication	none			none
Patients with aminosalicylates				
>3 g/day	–	7	2	–
2–3 g/day	–	–	1	–
Patients with steroids				
>40 mg/day	–	2	–	–
10–39 mg/day	–	1	–	–
<10 mg/day	–	1	1	–
Patients with immunosuppressive drug				
Azathioprine 100–150 mg/day	–	1 (+40 mg prednisone)	4	–

the sections were washed with washing solution (TBS/NaCl, Tween 0.05%) and incubated with normal horse serum for 30 min at room temperature as blocking solution. For BCRP staining, the tissue sections were incubated with a 1:1,240 dilution of the BCRP monoclonal antibody BXP-21 (Alexis Biochemicals, Lausen, Switzerland) overnight at 4°C or for Pgp staining with the Pgp monoclonal antibody ISB1-PGP with a 1:50 dilution. Samples were washed three times with washing solution and incubated with the horse antimouse IgG secondary antibody for 30 min at room temperature. After three washes with the washing solution, a perhydrol solution [H_2O_2 (0.3%), sodium azide (0.1%) in PBS] was used to destroy the endogenous peroxidase activity. The staining was performed with the avidin/biotinylated enzyme complex (ABC method) according to the manufacturer's instructions (Vectastain, Elite kit, Vector Laboratories, Burlingane, Calif., USA). For detection, 3-amino-9-ethylcarbazole, which forms a red end product, was used (Biogenex, San Ramon, Calif., USA). Sections which served as negative controls were treated equally, except that they were not incubated with the primary antibody.

Semiquantitative analysis of Pgp and BCRP staining was performed in biopsies of control subjects (Pgp n = 7; BCRP n = 4), of patients with newly diagnosed UC in inflamed (Pgp n = 4; BCRP n = 3) vs. unaffected tissue (Pgp n = 3; BCRP n = 3) as well as of patients with therapy-refractory UC in inflamed (Pgp n = 4; BCRP n = 3) vs. unaffected tissue (Pgp n = 3; BCRP n = 3) and of patients in remission (Pgp n = 6; BCRP n = 3). Rating of protein staining on blinded specimens was done by a trained pathologist. Expression levels were rated as follows: 0 = no expression, 1 = low, 2 = intermediate, 3 = high expression.

Statistics

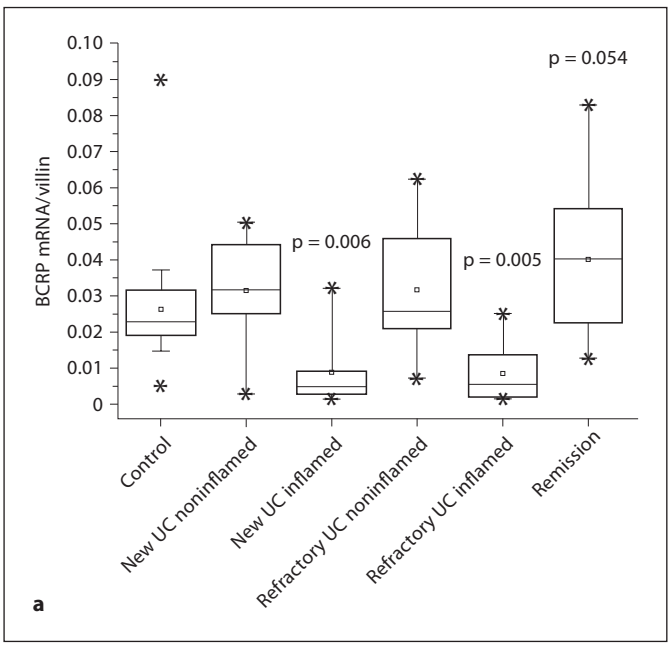
All values were expressed as means ± SEM. UC patients' MDR1 and BCRP expression was compared with that of control subjects by analysis of variance and subsequent Dunnett multicomparison test. All comparisons were performed as two-sided comparisons using the SPSS for Windows software (version 14.0). The level of significance was set at $p < 0.05$.

Results

BCRP and MDR1 mRNA Expression Is Downregulated in the Inflamed Mucosa of Patients with Active UC

BCRP, MDR1 and villin mRNA expression was analyzed in 26 control subjects and compared with patients with newly diagnosed UC (10 biopsy specimens from unaffected mucosa and 12 from inflamed mucosa), patients with active UC and refractory to anti-inflammatory medication (11 biopsy specimens from unaffected mucosa and 12 from inflamed mucosa) and UC patients in remission without any signs of acute disease.

BCRP in the inflamed mucosa of newly diagnosed untreated UC patients was reduced to 30% when compared with the unaffected mucosa (fig. 1a). Mean BCRP mRNA expression ± SEM was 0.009 ± 0.002 in the inflamed and 0.032 ± 0.005 in the unaffected mucosa. Similarly, in patients with active UC refractory to anti-inflammatory treatment, BCRP mRNA expression was significantly ($p = 0.006$) reduced to 35% in inflamed mucosa (0.009 ± 0.003), when compared with control subjects (0.026 ± 0.0029; $p = 0.005$). Patients treated with 5-aminosalicylates (n = 7) showed an 8-fold reduction in BCRP expres-



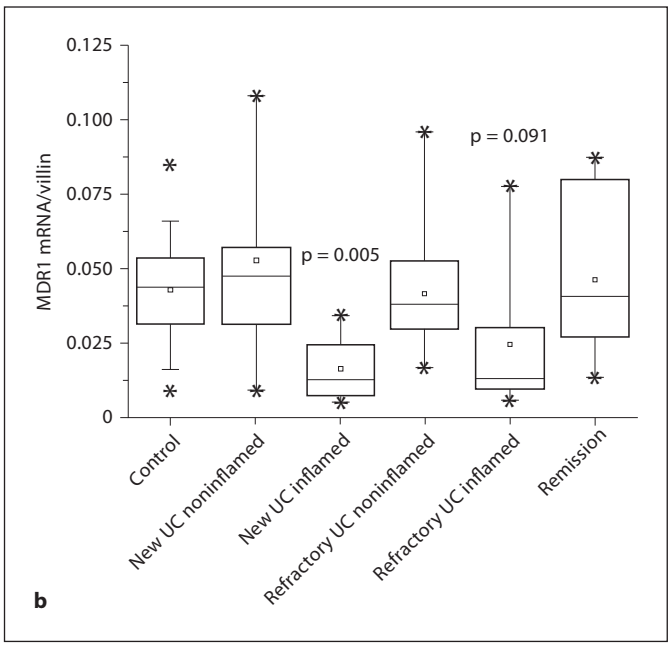


Fig. 1. a BCRP mRNA expression (BCRP/villin) in 26 healthy subjects, in colonic tissue sections with no visible inflammation of 10 newly diagnosed UC patients and in 12 acute inflamed tissue sections of newly diagnosed UC patients, in colonic tissue sections with no visible signs of inflammation of 11 therapy-refractory patients and in 12 acute inflamed tissue sections of therapy-refractory patients, in tissue sections of 17 UC patients in remission. Data represent means ± SEM. Statistical comparisons with controls were obtained. **b** MDR1 mRNA expression (MDR1/villin) in 26 healthy subjects, in colonic tissue sections with no visible inflammation of 9 newly diagnosed UC patients and in 11 tissue sections from acutely inflamed sites of newly diagnosed UC patients, in colonic tissue sections with no visible signs of inflammation of 11 therapy-refractory patients and in 12 tissue sections from acutely inflamed sites of therapy-refractory patients, and in tissue sections obtained from 17 UC patients in remission. Data represent means ± SEM. Statistical comparisons with controls were obtained.

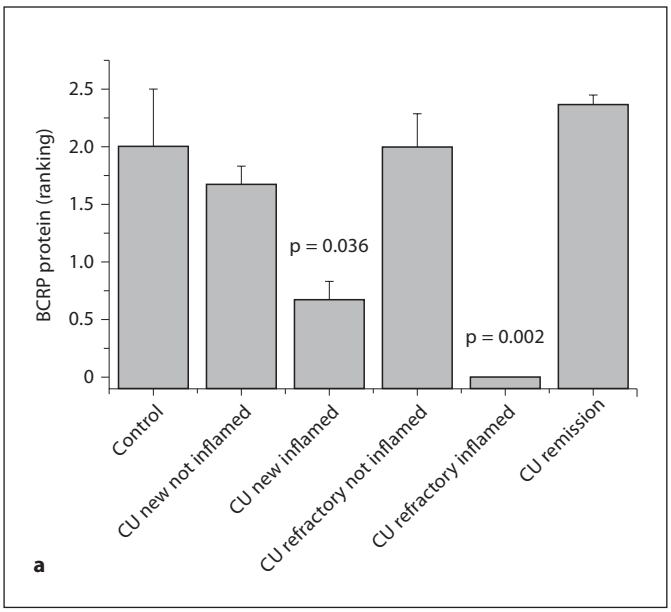
sion in the inflamed mucosa (0.005 ± 0.001) versus unaffected mucosa (0.04 ± 0.006), whereas only an insignificant decrease for prednisone-treated patients (n = 4) was observed.

Almost identical BCRP expression levels were observed in the unaffected mucosa of patients with active UC (newly diagnosed and refractory to anti-inflammatory medication) and in the unaffected mucosa of control subjects. BCRP mRNA levels of UC patients in remission (0.040 ± 0.006) were higher compared with control subjects. This increase was borderline significant (p = 0.054).

Similar findings were observed for MDR1 mRNA expression. In inflamed mucosa of patients with active UC, independent if newly diagnosed or refractory to treatment, MDR1 showed lower expression levels when compared with the unaffected mucosa (fig. 1b). In control subjects, mean MDR1 ± SEM expression was 0.043 ± 0.003, in newly diagnosed UC patients 0.016 ± 0.003 in the inflamed (p = 0.005) and 0.053 ± 0.011 in the unaffected mucosa. In therapy-refractory patients with the inflamed mucosa, mean MDR1/villin expression was 0.025 ± 0.007 (which was borderline significantly lower than in controls, p = 0.091) and 0.042 ± 0.006 in the unaffected mucosa. Again, patients treated with 5-aminosalicylates showed significantly reduced MDR1 expression in the inflamed mucosa, 0.011 ± 0.002 versus 0.037 ± 0.005 in the unaffected tissue. MDR1 expression in UC patients in remission (0.049 ± 0.008) was comparable to control subjects.

BCRP and Pgp Levels Are Reduced in the Inflamed Mucosa in Patients with Active UC

Expression of BCRP and Pgp was evaluated by immunohistochemistry in control subjects, newly diagnosed, and therapy-refractory patients with UC, as well as UC patients in remission. In the group of newly diagnosed and therapy-refractory patients, tissue samples obtained from inflamed mucosa and samples from unaffected mucosal sites were analyzed separately.



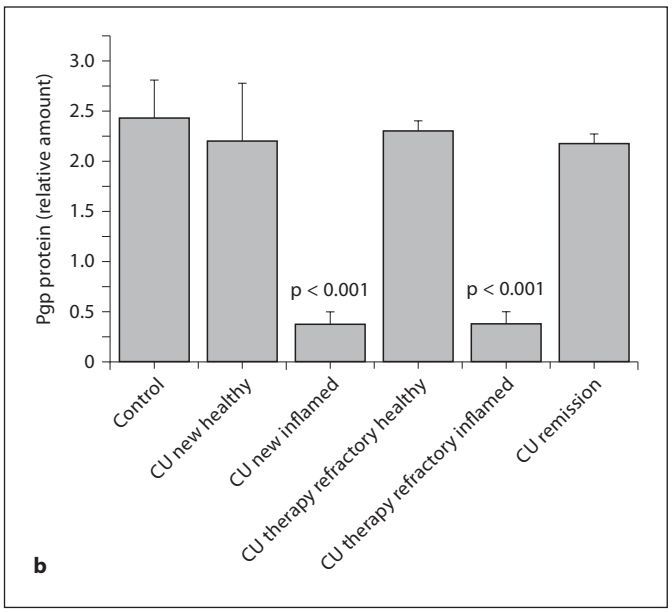


Fig. 2. a BCRP amount in selected healthy subjects, in colonic tissue sections with no visible signs of inflammation of 10 newly diagnosed UC patients and in 12 tissue sections obtained from acutely inflamed sections of newly diagnosed UC patients, in colonic tissue sections with no visible signs of inflammation of 11 therapy-refractory patients and in 12 tissue samples from acutely inflamed sections of therapy-refractory patients, in tissue sections of 17 UC patients in remission. Data represent means ± SEM. Statistical comparisons with controls were obtained. **b** Pgp amount in selected healthy subjects, in colonic tissue sections with no visible signs of inflammation of 10 newly diagnosed UC patients and in 12 tissue sections obtained from acutely inflamed sites of newly diagnosed UC patients, in colonic tissue sections obtained from sites with no visible signs of inflammation of 11 therapy-refractory patients and in 12 tissue sections obtained from acutely inflamed sites of therapy-refractory patients, in tissue sections of 17 UC patients in remission. Data represent means ± SEM. Statistical comparisons with controls were obtained.

As expected, both ABC transporter proteins were localized on the apical membrane of the epithelial cells (see fig. 3, 4). Pgp and BCRP were significantly reduced in inflamed mucosa of newly diagnosed as well as therapy-refractory patients. Protein expression in the unaffected tissue of control subjects was comparable with expression in unaffected tissue of UC patients with active disease as well as in UC patients in remission (fig. 2). In tissue sections from the inflamed mucosa, BCRP expression was reduced by 63% in newly diagnosed and was even completely abolished in therapy-refractory UC patients when compared with control subjects (fig. 2a). Similarly, Pgp expression was reduced by about 85% in the inflamed mucosa of patients with active UC compared with control subjects (fig. 2b).

Representative pictures of immunohistochemistry with staining for BCRP are displayed in figure 3 and for Pgp in figure 4.

Discussion

The results of this study demonstrate that expression of BCRP and MDR1 is downregulated in patients with UC on the transcriptional and protein levels, but only in the inflamed mucosa. On the other hand, both transporters show a similar expression level in gut biopsies from unaffected sites of patients with active UC, in samples from patients in remission and in biopsies from noninflamed parts of the gut of control subjects. The reduction in transporter expression in inflamed mucosa of untreated newly diagnosed UC patients was similar to that in patients who have failed treatment with 5-ASA or corticosteroids, which suggests that this reduction is largely dependent on the inflammation rather than the anti-inflammatory treatment.

Efflux transport proteins are important components of the intestinal barrier against bacterial toxins, carcinogens and drugs [3–7, 9–13]. Altered expression of transport proteins and/or altered function of these proteins

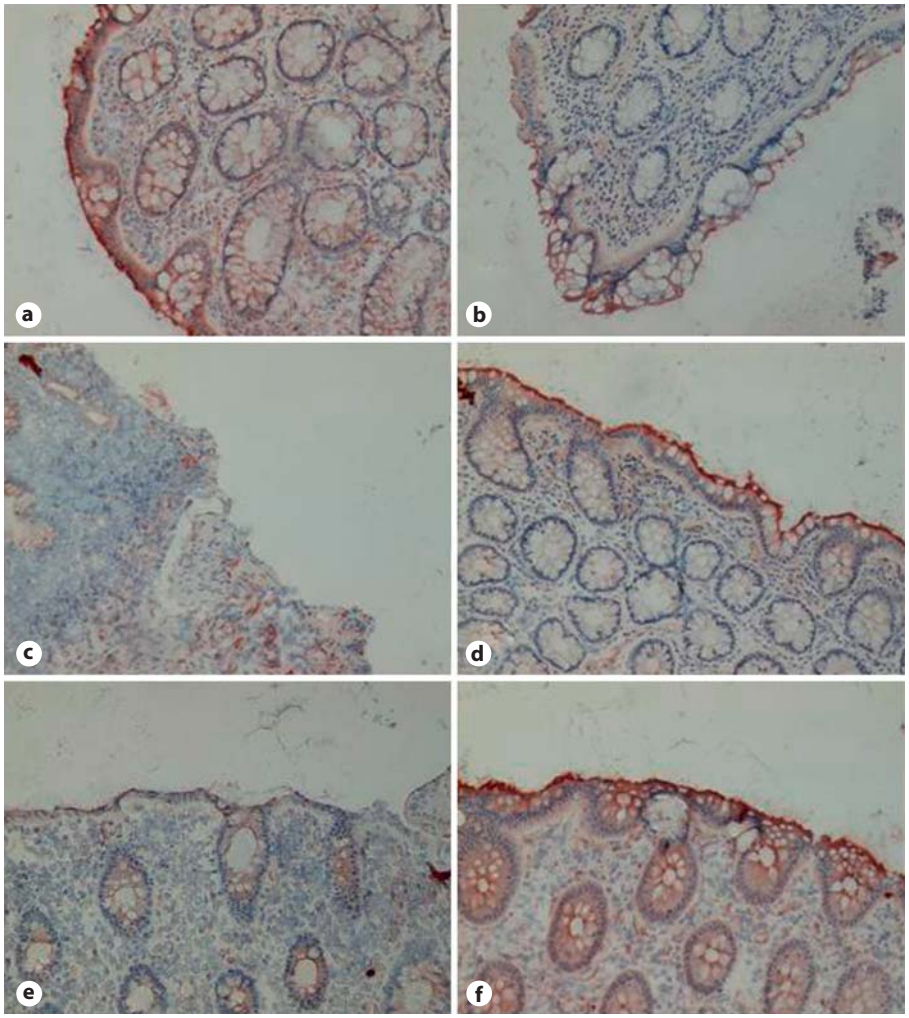


Fig. 3. Immunohistochemical staining of BCRP on the apical membrane of human duodenal epithelial cells using the BCRP antibody BXP-21. **a** Healthy subject. **b** Newly diagnosed UC patient, noninflamed tissue. **c** Newly diagnosed UC patient, inflamed tissue. **d** Therapy-refractory UC patient, noninflamed tissue. **e** Therapy-refractory UC patient, inflamed tissue. **f** UC patient in remission.

have been proposed to contribute to the pathogenesis of inflammatory disorders [16–21]. Here, we document that two of the efflux transporters of importance for the barrier function of the gastrointestinal mucosa, Pgp and BCRP, are expressed at markedly reduced levels in the inflammatory areas of the colon in patients with UC. This is in keeping with a recent study by Englund et al. [18] who also showed that BCRP and Pgp expression was reduced in inflamed tissue of UC patients that were taking anti-inflammatory drugs. We furthermore demonstrate that BCRP mRNA levels of patients with UC in remission are increased as compared with control subjects. These findings suggest that the inflammation itself might be responsible for the observed effects. Inflammation is known to suppress the expression and activity of several efflux transporters; also, the expression and function of intestinal mdr1 and mrp2 are reduced in a rat model of intestinal inflammation. Here, we extend these observations by showing that BCRP and MDR1 expression is reduced in the inflamed part compared with noninflamed tissue in individuals with active disease. Finally, the effect of drug treatment on the expression of these two transporters is related to their anti-inflammatory activity: patients that were treated with 5-aminosalicylates (n = 7) and did not respond to this treatment showed a considerable suppression in BCRP and MDR1 expression in the inflamed mucosa when compared with the unaffected area. On the other hand, patients treated with prednisone (n = 4) showed only an unremarkable and insignificant decrease in both transporters. Although prednisone was identified as a substrate for Pgp [27], it is not known to induce Pgp or BCRP. We conclude, therefore, that the reduced expression observed for the two transporters is a direct result of the inflammation. The find-

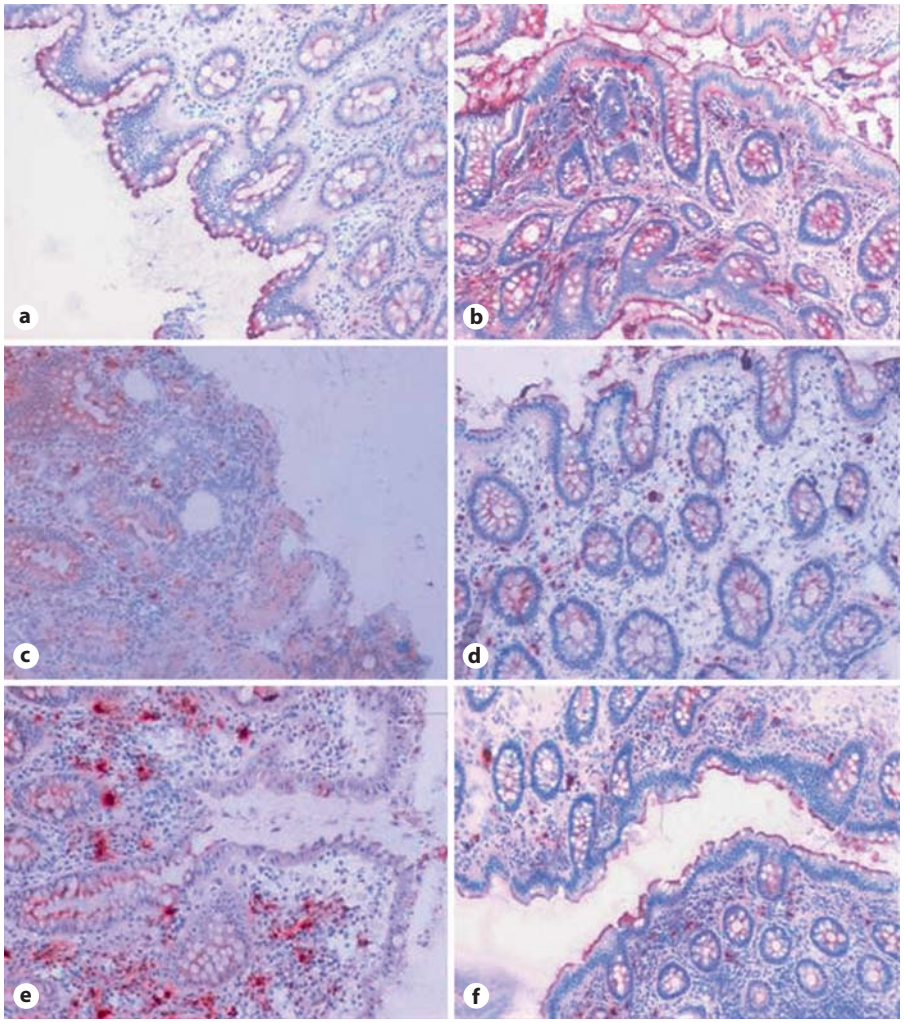


Fig. 4. Immunohistochemical staining of Pgp on the apical membrane of human duodenal epithelial cells using the Pgp antibody ISB1-PGP. **a** Healthy subject. **b** Newly diagnosed UC patient, noninflamed tissue. **c** Newly diagnosed UC patient, inflamed tissue. **d** Therapy-refractory UC patient, noninflamed tissue. **e** Therapy-refractory UC patient, inflamed tissue. **f** UC patient in remission.

ings furthermore suggest that the two transporters are most likely not involved in the pathogenesis of UC.

It is well known that the expression and activity of a number of hepatic efflux transporters such as MRP2, MRP3, MRP4, and organic anion-transporting polypeptides are diminished during inflammatory processes [28–30]. Alterations in transporter expression do not only occur in the liver, but also in kidney and intestine. Recently, we reported a decrease in BCRP expression in cholestatic patients [31] indicating that BCRP downregulation might be a phenomenon also observed in other gastrointestinal diseases. In vitro data indicate that the transcriptional regulation of BCRP is mediated via the Ah receptor [8], whereas Pgp is regulated by PXR [32]. Several proinflammatory cytokines have been shown to influence transporter gene expression [33, 34]. In fact, Evseenko et al. [35] found that incubation of primary term trophoblasts with TNF-α or IL-1β decreased mRNA and protein expression of Pgp and BCRP to 40–50%, respectively. They could also show that TNF-α affects BCRP expression also at a functional level leading to increased mitoxantrone accumulation [35]. The mechanisms leading to the observed downregulation have to be elaborated.

As both transporters are potent efflux pumps for a variety of toxic compounds, a diminished expression might disturb the intestinal barrier and the excretory function of the gut. A reduced intestinal expression of BCRP and Pgp in inflamed mucosal sites could substantially contribute to the accumulation of carcinogens and other harmful substances (bile acids) in enterocytes. This in turn might partly explain the observation that patients with UC have a higher risk of developing colorectal carcinomas [36, 37].

In conclusion, we have shown that patients with UC exhibit a decreased expression of BCRP and MDR1, both on the mRNA and protein levels in inflamed sites of the mucosa. The data together with published information suggest that the inflammatory process is responsible for the reduced levels. A major role in the pathogenesis of UC is unlikely. However, a decreased expression of these transporters might increase the accumulation of food-derived carcinogens and toxins and might influence the pharmacokinetics and effects of anti-inflammatory drugs used in the treatment of UC.

Acknowledgments

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