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Expression of cytochrome P450 and *MDR1* in patients with proctitis

Mari Thörn[1,3,], Niklas Finnström[2], Stefan Lundgren[2], Anders Rane[2] and Lars Lööf[1]

[1] *Centre of Clinical Research, Uppsala University, Central Hospital, SE-721 89 Västerås, Sweden.* [2] *Department of Laboratory Medicine, Division of Clinical Pharmacology, Karolinska institutet, Karolinska University Hospital (Huddinge), SE-141 86 Stockholm, Sweden.* [3] *Department of Medical Sciences, Division of Gastroenterology and Hepatology, University Hospital, SE-751 85 Uppsala, Sweden*

Abstract

Background. The aim of this study was to investigate the effect of inflammation on the gene expression of three cytochrome P450's (CYP) and P-glycoprotein (P-gp) in the rectal and colonic mucosa in patients with proctitis.
Methods. Biopsies were obtained from inflamed and normal mucosa in association with routine sigmoidoscopy in patients with proctitis. The biopsies were snap-frozen in liquid nitrogen. Real time PCR (polymerase chain reaction) was used for quantitative analyses of mRNA specific for the *CYP2E1, CYP3A4* and *CYP3A5* gene and the *MDR1* genes. Values were normalised based on gene expression of β-actin to enable comparisons between samples.
Results. The gene expression of CYP2E1 and CYP3A4 was lower in mucosa with severe inflammation vs normal mucosa ($p<0.05$). For CYP3A5 and P-gp there was no significant difference when comparing normal and inflammatory changed mucosa.
Conclusion. Our study suggests that at least for some of the CYP enzymes the expression decreases in response to the inflammatory process in the gastrointestinal tract.

Introduction

The cytochrome P450 (CYP) system is a supergene family of enzymes, which are responsible for detoxification, and metabolism of drugs and other xenobiotics. This system is also of major importance for a number of endogenous metabolic processes that include synthesis of steroid hormones, bile acids and metabolism of fatty acids [1].

The multidrug resistance gene (*MDR1)* is coding for P-glycoprotein (P-gp) which is another important determinant of oral bioavailability of many drugs and other xenobiotics. P-gp pumps certain drug substrates from the epithelial gut cells back into the lumen [2] thereby lowering intracellular concentration of its substrates [3].

Even though the liver is the most important organ for detoxification of foreign compounds it has been clear over the recent years that the gastrointestinal (GI) tract may contribute significantly in the pre-systemic metabolism of orally administered drugs [4]. Among other CYP enzymes CYP2E1, CYP3A4, CYP3A5 have been described in the gastrointestinal mucosa «[5,6,7,8]».

Ulcerative colitis and Crohn's disease are inflammatory bowel diseases (IBD) of unknown pathogenesis. Ulcerative colitis affects only the colon but Crohn's disease

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can affect the whole gastrointestinal tract from mouth to anus and the inflammation in Crohn's disease involves deeper parts of the mucosa [9]. In proctitis, inflammation is confined to the rectal mucosa and the most usual form is ulcerative proctitis even though Crohn's disease sometimes causes isolated proctitis.

Proctitis is usually treated with topical enemas or suppositories containing steroids or 5-acetyl salicylic acid. It is known that some patients with proctitis do not respond to the treatment [10]. High activity of P-gp may be one contributing factor for this as it has been shown that patients with Crohn's disease that express higher levels of P-gp more often fails to respond to glucocorticoid therapy [11].
Since CYP and P-gp are polymorphically expressed «[12–13]», it is plausible that the local expression of CYP and P-gp can influence the variation in response to treatment. It is also possible that the inflammation itself alters the expression of these proteins.

It is known that inflammation decreases the level and activity of most CYP enzymes at least in the liver. However, the level of reduction by inflammation can vary between the different CYP enzymes since they appear to be regulated by different cytokines [14]. Furthermore, little is known about the effects of inflammation on CYP expression in extrahepatic tissues such as the gastrointestinal mucosa [15]. A decrease in expression of these enzymes may influence the metabolic capacity. Moreover, it was shown in a study with the human colon carcinoma cell line (Caco-2 cells) that cytokines influence the mRNA expression of CYP3A4 and *MDR1* such that the expression of CYP3A4 decreases but *MDR1* gene expression increases [16].

Panwala et al. showed that *mdr1a* knockout mice develop colitis which can be prevented by the use of antibiotics [17]. This has raised the question whether reduced expression or activity of the *MDR1* gene may be a contributing factor in the pathogenesis of IBD [18]. In a study by Schwab et al [19] it has been claimed that there is an association between the C3435T *MDR1* gene polymorphism and susceptibility to ulcerative colitis. However, other authors were unable to repeat this finding «[20–22]», [2], Another polymorphism of *MDR1*, Ala 893, has also been proposed to be associated with IBD [13].

Given this background the aim of this study was to investigate if the gene expression of *CYP2E1, CYP3A4, CYP3A5* and *MDR1* in patients with proctitis is different in inflammatory changed versus normal mucosa using real time RT-PCR, [23]

Patients and methods

Patients presenting with symptoms of frequent, bloody stools at the endoscopy unit, University hospital of Uppsala, were asked to participate in the study. Patients were included if they had macroscopic inflammation in the rectum with a clear limitation to normal mucosa in the sigmoid colon. The Ethics committee of the University approved of the project. After informed consent, biopsies were obtained from inflamed mucosa in the rectum and adjacent macroscopically normal mucosa in more

proximal part of the bowel from 11 patients. The inflammation was characterized clinically by the endoscopist as severe or mild. Severe was defined as red, swollen mucosa with contact bleeding and/or ulcerations. The biopsies were snap-frozen in liquid nitrogen and then stored at -70°C until preparation of mRNA. A record was taken concerning the patients smoking and alcohol habits and medication the week before the investigation. The clinical data of the patients is shown in Table 1.

mRNA purification

The Quick prep micro mRNA purification kit (Amersham Biosciences, Sweden) was used for purification of the mRNA according to the instructions from the manufacturer.

After purification, mRNA was precipitated in 95% ethanol at -20°C for 1 hour and then resuspended in 50 µl RNAse-free water. The amount of mRNA was measured at 260 nm, with correction for background at 320 nm.

cDNA-synthesis

Thirty-five or 40 ng of purified mRNA was used for cDNA synthes with "First strand cDNA synthesis kit" (Amersham Biosciences, Sweden).For two of the 22 samples less than 35 ng mRNA was obtained. This was corrected for in the calculations when needed.

Table 1. Clinical features of the patients

Patient	Age	Sex	Smoking	Alcohol	Medication	Clinical diagnoses
1	28	m	No	No	None	Ulcerative proctitis
2	45	m	No	10g	None	Ulcerative proctitis
3	30	f	No	No	None	Ulcerative proctitis
7	25	m	0-1/d	173g	None	Crohn's proctitis
9	29	f	No	5g	mesalazine orally	Ulcerative proctitis
10	43	f	No	7g	steroid enema	Ulcerative proctitis
11	28	f	No	77g	sulphasalazine orally, mesalazine topically	Ulcerative proctitis
12	30	m	3-5/d	10g	steroids,mesalazine orally	Ulcerative proctitis
15	32	m	No	No	None	Ulcerative proctitis
16	78	m	No	No	sulphasalazine orally, mesalazine topically, steroid enema, levodopa	Ulcerative proctitis
17	34	m	No	90g	steroids, sulpha-salazine orally	Crohn's proctitis?

Real-time PCR

The cDNA was diluted 1:10 with Millipore-filtered water before use in the real-time RT-PCR reaction. Primer pairs and fluorescent Taqman™ probes specific for *CYP2E1, CYP3A4, CYP3A5* and *MDR1* were used and designed as described earlier «[24–25]». The house keeping β-actin gene was used as an external standard to enable comparison between samples. The β-actin probe was labelled with FAM (6-carboxyfluorescin) and purchased from Applied Biosystems.

Ten µl of the diluted cDNA mix, containing 3.3 ng mRNA, 300 nM each of forward and reverse primers, 50 nM of the probe and a ready-made mastermix with heat-activated Taq polymerase, uracil-N-glycosylase (UNG) and nucleotides (Applied biosystems, Sweden) were used in the PCR reaction.

Real-time PCR was performed on an ABI PRISM™ SDS 7700 (Perkin-Elmer). An initial step for UNG degradation, UNG inactivation for 2 min at 50ºC and a Taq polymerase activation step at 94ºC for 10 min were followed by 50 cycles (each at 95ºC 15 s; 60ºC 30 s). All samples were run in duplicates. At least two non-template controls were included in all PCR runs.

The cycles to threshold (Ct) value was determined in which the different PCR reactions were in early logarithmic phase. Limit of detection for the system was defined in an earlier study [25]at a Ct value of 38.

Ct values of duplicates varied between 0 and 74% with a median of 1.2% and a mean of 3.4%.

Statistics

Statistical analyses were executed by the use of Statistica® (StatSoft Inc., USA) and SPSS® SPSS Inc, USA) Non-parametric tests were used to analyse the data. For comparison of independent samples, the Mann-Whitney test was used. For comparison between dependent samples, the Friedman ANOVA with Kendall coefficient of variation and the Wilcoxon matched pair test were used.

Results

The percentage of biopsies with Ct values considered below the predetermined cut-off value of 38 varied between the different enzymes. CYP2E1 was expressed in 90% of the non-inflamed biopsies and in 81% of the inflamed biopsies. For CYP3A4 the expression was below detection limit in the majority of the samples and it was expressed in 45% and 18% in the non-inflamed vs. inflamed biopsies, respectively. CYP3A5 however, was expressed in 100% of the samples. For P-gp the expression rate was 90% in both non-inflamed and inflamed samples. However, since all samples expressed β-actin the statistical calculations were performed including all samples.

The expression of the enzymes is shown in Table 2. CYP2E1 was expressed at a significantly higher level than the other investigated enzymes ($p<0.0001$ Friedman ANOVA) both in biopsies from non-inflamed and inflamed mucosa. The inter-indi-

vidual variation was low (Kendalls coefficient of variation 0.85). CYP3A4 was the enzyme with the lowest expression.

The expression levels in inflamed vs. macroscopically normal mucosa did not differ when comparing all patients (Wilcoxon matched pair test). However, an analysis of a subgroup of 5 patients with more severe macroscopic inflammation (red swollen mucosa, ulcerations and/or contact bleeding) showed lower expression of CYP2E1 and CYP3A4 in inflamed compared to normal mucosa ($p<0.05$ Wilcoxon matched pair test). For CYP3A5, four of the five patients with severe inflammation had lower expression level but this was not statistically significant ($p=0.08$, Wilcoxon matched pair test). The expression of P-gp was also lower in inflamed mucosa in four of these five patients albeit not significant ($p=0.5$ Wilcoxon matched pair test). See Figure 1.

Table 2: CYPspecific mRNA/β-actin specific mRNA ratio: median (Q25-Q75) (all patients)

	CYP2E1	CYP3A4	CYP3A5	P-gp
Non-inflamed	0.6731 (0.1110-1.3382)	0.0004 (0,00011-0,0020)	0.0027 (0.0018-0.0051)	0.0014 (0.0004-0.0025)
Inflamed	0.4996 (0.3010-1.5567)	0.00004 (0.00001-0,0002)	0.0034 (0.0010-0.0044)	0.0003 (0.0002-0.0010)

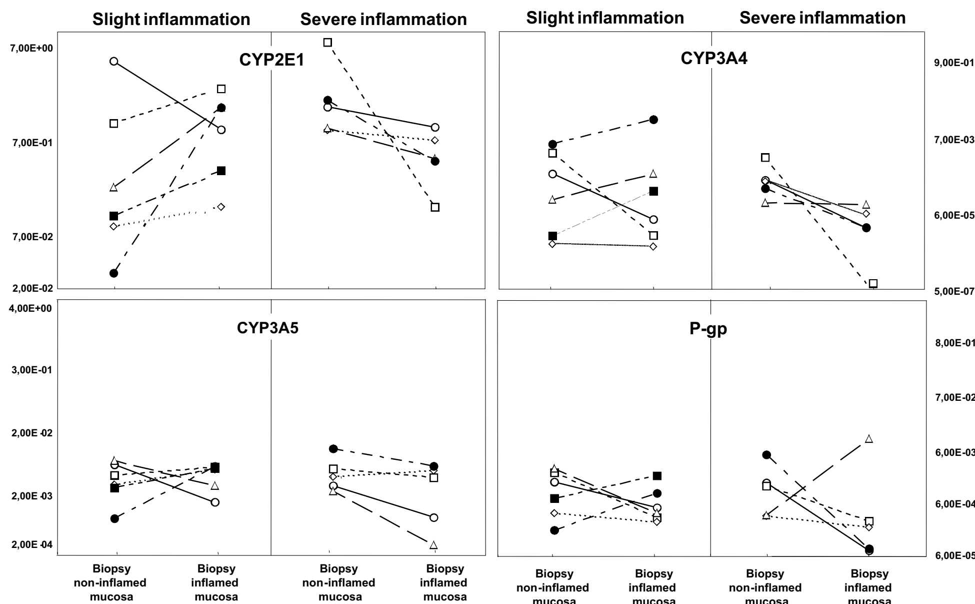

Figure 1. Subgroup analyses of 5 patients with more severe macroscopic inflammation (red, swollen mucosa, ulcerations and/or contact bleeding) showed lower expression of CYP2E1 and CYP3A4 in inflamed compared to normal mucosa ($p<0.05$, Wilcoxon matched pair test). For CYP3A5, 4 of the 5 patients with severe inflammation had lower expression level but this was not statistically significant ($p=0.08$, Wilcoxon matched pair test). The expression of P-gp was also lower in inflamed mucosa in 4 of these 5 patients albeit not significant ($p=0.5$ Wilcoxon matched pair test).

There was no significant difference between the patients in respect of smoking habits, alcohol intake, or medication (Mann-Whitney test).

Discussion

Our hypothesis was that the expression of the investigated CYP enzymes and P-gp would be decreased as a result of the mucosal inflammation. This was the case for CYP2E1 and CYP3A4 but only in patients with severe inflammation. For CYP3A5 there was a trend towards decreased expression in the group of patients with more severe inflammation, although not significant. This may be due to the small number of patients, at least with severe inflammation. Another possible explanation is that the individual CYP enzymes are regulated differently in inflamed gastro-intestinal mucosa than in the liver [15].

The present data confirmed our earlier observation that CYP2E1 is the CYP enzyme with the highest expression in the gastrointestinal mucosa [8]. As this enzyme is post–transcriptionally regulated [26] several authors have failed to find a relation between mRNA and protein level «[27–28]».

CYP3A4 had the lowest expression of the investigated enzymes in the rectal and colonic mucosa which is also in agreement with our previous data [8] and with earlier findings on human transplant donors «[6–7]». It has been shown in Caco-2 cells that inflammation decreases mRNA expression of CYP3A4, increases epithelial permeability and increases expression of *MDR1*. This suggests that inflammation may affect bioavailability even though the increase in P-gp may counteract the first two effects [16].

As previously shown by our group [8] CYP3A5 mRNA was measurable in 100% of the samples. The expression of this enzyme is polymorphic, and individuals with at least one CYP3A5*1 allele express greater amounts of the enzyme [29]. However, our data suggest that the expression of CYP3A5 mRNA is monomorphic since the CYP3A5*3 allelic variant also expresses mRNA to a varying degree. This enzyme has been shown to be expressed in the colon also by other authors «[6-7]»., It has been suggested that CYP3A5 may be a very important contributor to CYP3A mediated metabolism in the intestines [30]. It is hard to find any published data of the effects of inflammation on CYP3A5 expression for comparison. It was a tendency to lower expression of CYP3A5 in patients with more severe proctitis.

The *MDR1* gene expression was almost of the same order of magnitude as CYP3A5. There are some data that p-gp is expressed at relatively high levels in the distal colon «[8,31–32]». It has been shown that inflammation induced in rat livers decreases the expression of P-pg [33]. For intestinal mucosa, however, earlier data are in conflict with the present results, perhaps as a result of different methodology. For example, treatment with the proinflammatory cytokines IL-1 and IL-6 resulted in a decrease of P-gp mRNA expression, protein expression and activity in animal models [34]. In contrast, Bertilsson et al. have shown that these cytokines increase the expression of *MDR1* in the human Caco-2 intestinal cell line [16]. It seems that

different tissues and species respond differently to inflammation with regard to *MDR1* expression. A previous study (33) using an immunohistochemical method in surgical samples from patients with ulcerative colitis reported that the percentage of patients expressing P-pg was higher in inflamed mucosa compared to macroscopic normal mucosa in the same patient. However, there were some contradictory observations in that study, which are difficult to explain. There was for example no difference between inflamed and normal areas in patients with duration of disease over 5 years and patients who had long-standing inactive colitis more frequently expressed P-gp but only within histological normal areas [35]. In our present study, there was no significant difference in P-gp expression in normal compared to inflamed mucosa. However, the median value of P-gp expression was about 5 times less in inflamed mucosa and was lower in four of five patients with severe inflammation. This trend is in disagreement with results of Saclarides et al. [35] and Bertilsson et al. [16]. One possible explanation is that Saclarides et al., studied protein expression evaluated by immunohistochemistry whereas we have compared the gene expression of the enzymes and P-gp. Thus, there may be a discrepancy between the protein level and the gene expression. The samples available in this clinical situation were not sufficient for protein or enzyme activity analyses.

Another potential explanation is that the study by Saclarides et al. [35] was performed in patients where many (10 of 21) had dysplasia. In our study, no patient had dysplasia. The suggestion that malignant transformation may upregulate the expression of P-gp is in agreement with data from a recent study where higher differentiation resulted in down-regulation of P-gp in the Caco-2 cell line [36] which was the cell line used by Bertilsson et al. [16]

The mutual expression pattern of these enzymes is similar to a study from our group where most patients had IBS [8]. However, the expression was higher in this study for all enzymes except CYP3A5.

We found no differences between patients that were related to smoking habits, alcohol intake, or medication. However, as the groups were very small for individual drugs this is not expected and the finding has to be confirmed.

In conclusion, we found a difference in gene expression of CYP2E1 and CYP3A4 between macroscopically normal and severely inflamed colonic mucosa, with a decrease in expression in the inflamed mucosa. For CYP3A5 and P-gp the difference was not significant, although there was a trend towards a lower expression in patients with more severe proctitis. This suggests that like the case with liver, inflammation also decreases the expression of CYP enzymes in the gastrointestinal mucosa. This may influence the response to treatment of bowel inflammation.

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Corresponding author:
Mari Thörn
Medical Products Agency,
Box 26
SE-751 03 Uppsalas, Sweden
E-mail: Mari.Thorn@mpa.se
Tel + 46-18-17 49 68
Fax: +46-18-54 85 66