P-Glycoprotein Increases from Proximal to Distal Regions of Human Small Intestine

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Purpose. The contribution of the efflux transporter P-glycoprotein (P-gp) as a barrier to drug absorption may depend on its level of expression at the site of absorption. Accordingly, the distribution of P-gp was examined along the entire length of the human small intestine.

Methods. Homogenates prepared from mucosal scrapings from every other 30-cm segment of four unrelated human donor small intestines were analyzed for P-gp and the control protein villin by Western blot.

Results. In each donor intestine, relative P-gp expression (P-gp/villin integrated optical density ratio) progressively increased from proximal to distal regions. Among individuals, relative P-gp levels varied 2.1-fold in the duodenal/proximal jejunal region, 1.5- to 2.0-fold in the middle/distal jejunal region, and 1.2- to 1.9-fold in the ileal region. Within-donor variation was somewhat greater, from 1.5- to 3.0-fold.

Conclusions. These results provide further evidence that the site of absorption can represent another source for the interindividual variation in the oral bioavailability of drugs.

KEY WORDS: P-glycoprotein; human; intestine; absorption; drugs.

INTRODUCTION

Although presystemic metabolism in the liver is a well-established source for the incomplete absorption and low oral bioavailability of drugs, accumulating evidence indicates that the small intestine can represent a second presystemic site of metabolism (1,2). Several of the drug-metabolizing enzymes expressed in the liver are also expressed in the small intestine and include the UDP-glucuronosyl transferases, sulfotransferases, glutathione S-transferases, N-acetyl transferases, and the cytochromes (CYP) P450 (1,3). *In vivo* studies in humans have demonstrated that the contribution by the intestine to overall first-pass metabolism may even rival that by the liver for some drugs. Midazolam, verapamil, and nifedipine are such examples (reviewed in reference 1), all of which are substrates for CYP3A4, the major CYP isoform expressed in adult human liver and small intestine, at least in most Caucasians (4–7). This enzyme, believed to be involved in the metabolism of roughly half of currently prescribed drugs (8), exhibits a high degree of interindividual variation. Both its expression and its catalytic activity vary at least an order of magnitude not only in the liver but also in all three regions of the small intestine (i.e., duodenum, jejunum, and ileum) (6,9). This wide variation in enzyme expression and activity in both organs thus accounts in part for the large range in the oral bioavailabilities commonly observed for a number of CYP3A4 substrates (8). Moreover, CYP3A4 protein levels and catalytic activity are generally highest in proximal small intestine, then progressively decline toward distal ileum (6,10), suggesting that the site of absorption can represent another source for the interindividual variation in the oral bioavailability of CYP3A4 substrates.

In addition to metabolizing enzymes, a role for transport proteins in limiting the oral bioavailability of drugs has become increasingly recognized (11). Of these transporters, the active secretory (efflux) pump P-glycoprotein (P-gp) is the most widely studied in terms of drug disposition. P-gp, a 170-kd transmembrane glycosylated protein and gene product of *MDR1*, has been identified in a variety of tissues, including the epithelial cells lining the small intestine (enterocytes) (12). Because of its location on the apical (luminal) membrane of enterocytes, P-gp acts to reduce the absorption of its substrates, many of which are also metabolized by CYP3A4. Such drugs include the immunosuppressants cyclosporine and tacrolimus, the HIV protease inhibitors saquinavir and indinavir, and the chemotherapeutic agents paclitaxel and vinblastine (13). Because CYP3A4 and P-gp are located in close proximity in the mature enterocytes lining the villi, it has been proposed that these two proteins act in concert to reduce drug absorption and oral bioavailability (13,14).

P-gp was shown to be variably expressed among healthy people, from two- to fourfold, when measured in duodenal pinch biopsies by Western blot analysis (15–17); unlike CYP3A4, however, less is known about P-gp expression in more distal regions of the small intestine. Until now, only *MDR1* mRNA expression had been reported for the jejunum (18) and ileum (19). Moreover, because these results were reported by two different laboratories (using two different methods of analysis), it is difficult to compare relative levels of mRNA between the two regions. Therefore, to further elucidate the importance of the site of absorption in the interindividual variability in drug disposition, we characterized the relative expression of P-gp along the entire length of four human small intestines obtained from unrelated donors.

MATERIALS AND METHODS

Materials and Chemicals

Sodium dodecyl sulfate, acrylamide/bis (37.5:1), ammonium persulfate, and TEMED were purchased from Bio-Rad (Hercules, CA). The polyvinylidene difluoride (PVDF) membrane (Hybond-P) and enhanced chemiluminescence reagents were purchased from Amersham Biosciences, Inc. (Piscataway, NJ). A rabbit polyclonal antibody raised against a peptide of human P-gp was a kind gift from Dr. Erin G. Schuetz (St. Jude's Children's Research Hospital, Memphis, TN). [The peptide was identical to that used by Oncogene Research Products (Boston, MA) to prepare its rabbit polyclonal antibody, *mdr* (Ab-1). The antibody provided by Dr. Schuetz is more concentrated and exhibits less background than *mdr* (Ab-1).] A murine monoclonal antibody raised

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ABBREVIATIONS: *CYP*, cytochrome P450; *P-gp*, P-glycoprotein; *PVDF*, polyvinylidene difluoride; *1α,25-(OH)$_2$-D$_3$*, 1α,25-dihydroxyvitamin D$_3$; *IOD*, integrated optical density

against human villin was purchased from Chemicon International (Temecula, CA). The secondary antibodies, rabbit antimouse and goat antirabbit horseradish peroxidase–conjugated IgG, were purchased from Zymed Laboratories (South San Francisco, CA). All other reagents were of electrophoresis grade.

Human Tissue Homogenates

Homogenates previously prepared from mucosal scrapings from every other 30-cm segment of four full-length unrelated human donor small intestines and from a representative liver (6) were used for the present study. (The remaining intestinal segments were given to other investigators for unrelated studies.) Medical and drug histories of each donor are presented in Table I. Details of organ procurement and preparation of homogenates are described elsewhere (6). Based on the approximate lengths of each region of the human small intestine (20), the first 30-cm segment of each donor was designated duodenum/proximal jejunum (nos. 1 or 2), the next three segments as middle/distal jejunum (nos. 3, 5, 7 or 4, 6, 8), and the remaining segments as ileum. The Caco-2 cell homogenate was from a previous study (21) and was prepared from a monolayer treated with the CYP3A4/P-gp inducing agent, 1α,25-dihydroxyvitamin D_3 [1α,25-$(OH)_2$-D_3] (22). Total protein concentrations of all homogenate samples were determined by the method of Lowry *et al.* (23) using bovine serum albumin as the reference standard.

Western Blot Analysis

Because of the limited quantities of intestinal mucosal homogenates, samples were analyzed once (HIs 31, 32) or twice (HIs 35, 40). All homogenate samples (15 μg) were diluted in sample buffer, loaded onto 0.1% sodium dodecyl sulfate-7% polyacrylamide gels, and the proteins electrophoretically separated as previously described (21). The proteins were then transferred to PVDF membranes, and the membranes were probed for P-gp and the control enterocyte protein, villin, as described (21). The proteins of interest were visualized using enhanced chemiluminescence reagents and the Chemi-Doc imaging system (Bio-Rad). Integrated optical densities (IODs) were obtained using the Bio-Rad software program Quantity One (v4.2). Relative P-gp levels were expressed as the ratio of the IOD of P-gp to that of villin. To compare the distribution pattern of P-gp among individuals, the IOD ratios within each donor intestine were normalized to the maximum value, which was set to 1.00.

RESULTS AND DISCUSSION

Optimizing the oral route for drug administration is particularly relevant for chronically ill patients requiring long-term treatment with narrow therapeutic index drugs that exhibit large interindividual variation in oral bioavailability. Many such drugs are substrates for CYP3A4 and include those used in the prevention of organ transplant rejection and in the treatment of AIDS and various forms of cancer. Because CYP3A4 expression and catalytic activity tend to decrease from the proximal to distal regions of the small intestine, it has been suggested that if an orally administered CYP3A4 substrate is primarily absorbed in the distal region, its extent of first-pass metabolism would be lower than if absorbed in the proximal region (6). Thus, it was surmised that, compared with an immediate-release preparation, an extended-release preparation may be spared a significant first-pass effect in the intestine and have a greater oral bioavailability (6). Results from the present study, however, indicate this would not be the case if the drug were also a P-gp substrate.

Based on limited reports suggesting that *MDR1* mRNA expression increases from proximal to distal small intestine (18,19), we sought to determine whether this pattern of expression exists at the protein level, utilizing mucosal homogenates prepared from four full-length human donor small intestines. Compared with homogenate prepared from a 1α,25-$(OH)_2$-D_3-treated Caco-2 cell monolayer, our positive control for P-gp and villin expression, immunoreactive P-gp was faint to undetectable in the representative liver homogenate but was readily detected in all intestinal mucosal homogenates (Figs. 1 and 2) Similarly, whereas villin immunoreactive protein was not detected in the liver homogenate, it was readily detected in the intestinal homogenates; moreover, this structural protein remained relatively constant throughout the small intestinal tract (Figs. 1 and 2). The monoclonal antivillin antibody also recognized a second, lower-molecular-weight protein in the human intestinal mucosal homogenates but not in the Caco-2 homogenate, consistent with recent reports from this laboratory (21) and from others (16,24). The identity of this protein remains to be determined. In all four donor small intestines, relative P-gp expression (i.e., normalized P-gp/villin IOD ratio) followed the same general pattern, increasing progressively from proximal to distal regions (Table II, Fig. 3). Repeat analyses of HI-35 and HI-40 (Fig. 2B) revealed similar ascending patterns of expression, with the lowest level in the most proximal segment (0.52 and 0.73, respectively) and the highest level in the most (or second most) distal segment; jejunal values ranged from 0.52 to 0.69

Table I. Organ Donor Characteristics

Donor code*	Sex	Age (yr)	Ethnicity†	Medications	Disease	Cause of death
HI-31	M	48	C	None	Diabetes	Intracranial bleed
HI-32	M	16	A	None	None	Gunshot to head
HI-35	M	16	H	Unknown	Asthma	Motor vehicle accident
HI-40	F	21	C	None	None	Unknown
HL-152	F	64	C	Premarin®	Unknown	Intracranial bleed

* HI, human intestine; HL, human liver.
† C, Caucasian; A, Asian; H, Hispanic.

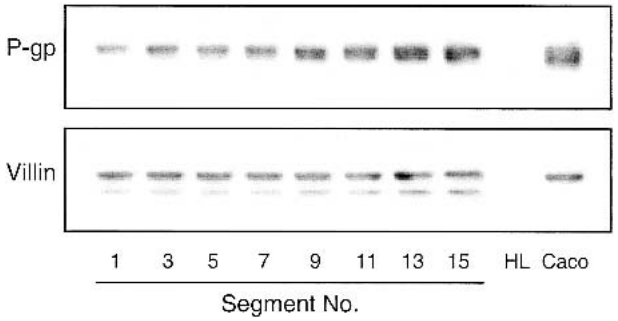

Fig. 1. Western blot showing the expression of P-glycoprotein and the control protein villin along the length of a human donor small intestine (HI-31). Segment 1 represents duodenum; segments 3, 5, and 7 represent middle to distal jejunum; and the remaining segments represent ileum. All segments measured approximately 30 cm in length. For a given intestine, the same blot was probed for both proteins, but optimal visualization of each required a different exposure time. HL, human liver homogenate. Caco, homogenate prepared from a representative Caco-2 cell monolayer treated with the CYP3A4/P-gp inducing agent $1\alpha,25\text{-}(OH)_2\text{-}D_3$. All lanes of the gel were loaded with 15 μg homogenate protein.

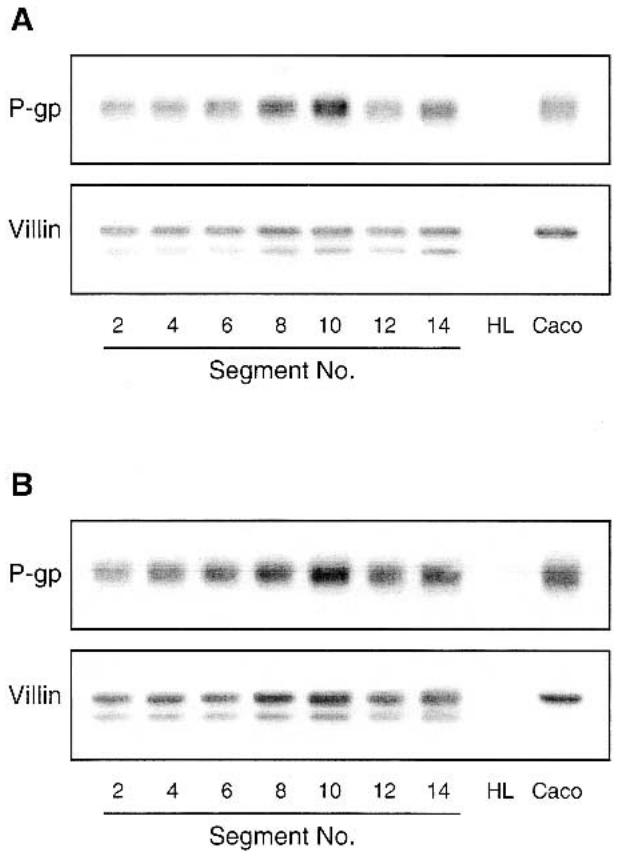

Fig. 2. Western blots showing the expression of P-glycoprotein and the control protein villin along the length of HI-40 on the first (A) and repeat (B) analyses. Segment 2 represents proximal jejunum; segments 4, 6, and 8 represent middle to distal jejunum; and the remaining segments represent ileum. All segments measured approximately 30 cm in length. Abbreviations and the amount of protein loaded are the same as those described for Fig. 1.

Table II. Relative P-gp Expression* Along the Length of Four Human Donor Small Intestines

Segment†	Donor code‡			
	HI-31	HI-32	HI-35	HI-40
Duodenum/proximal jejunum				
1/2	0.51	0.63	0.33	0.68
Middle to distal jejunum				
3/4	0.61	0.61	0.51	0.85
5/6	0.61	0.81	0.50	0.99
7/8	0.70	0.72	0.57	0.88
Ileum				
9/10	0.89	0.72	0.50	0.97
11/12	0.92	0.74	0.69	1.00
13/14	0.86	0.73	0.80	0.91
15/16	1.00	0.86	0.59	
17/18		1.00	1.00	
20		0.89		

* P-gp/villin IOD ratio (normalized to the maximum value).
† Each segment measured approximately 30 cm in length.
‡ HI, human intestine.

and from 0.74 to 0.95, and ileal values ranged from 0.60 to 1.00 and from 0.93 to 1.00, respectively. For this small sample size, sex, age, ethnicity, and concomitant medications/disease (Table I) appeared not to influence this trend.

The ascending pattern of P-gp expression in the small intestinal tract suggests that drug substrates for P-gp (but not CYP3A4), if primarily absorbed in the distal region (and if at subsaturating concentrations for efflux), would have a lower extent of absorption than if absorbed in the more proximal region. Indeed, in a healthy volunteer study involving the P-gp–nonmetabolized substrate talinolol, the area under the concentration vs. time curve (AUC) following distal instillation averaged 50% (range 15–73%) of that following proxi-

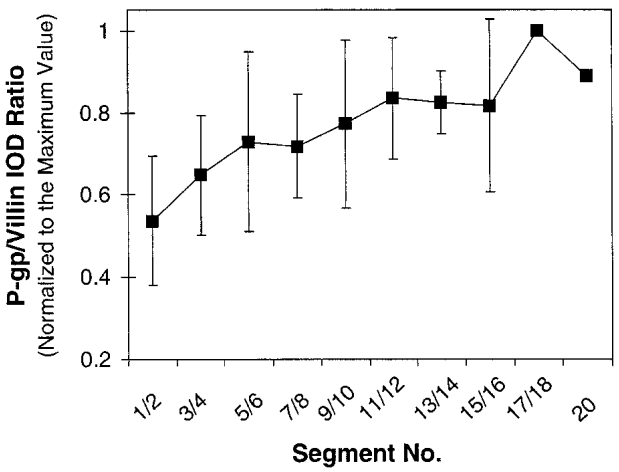

Fig. 3. Average and variation in P-gp content (P-gp/villin IOD ratio, normalized to the maximum value) for each segment of the human small intestine. Segment 1/2 represents the duodenum/proximal jejunum; segments 3/4, 5/6, and 7/8 represent middle to distal jejunum; and the remaining segments represent proximal to distal ileum. All segments measured approximately 30 cm in length. Squares and error bars denote means and SDs, respectively, of all four (segments 1/2 to 13/14) or three (segments 15/16) intestines examined. The square for segment 17/18 denotes the mean of two intestines, and that for segment 20 denotes the value for a single intestine.

mal instillation of the drug into the small intestine (25). However, an inverse correlation was also reported between gastrointestinal *MDR1* mRNA levels (colon > jejunum > stomach) and the AUC of the dual P-gp/CYP3A4 substrate cyclosporine following instillation of the drug into corresponding regions (stomach > jejunum/ileum > colon) (26). This apparent anomaly can be explained by the subsequent finding that variation in intestinal P-gp (and hepatic CYP3A4), but not intestinal CYP3A4, significantly accounted for the variation in both the maximum concentration and apparent oral clearance of cyclosporine (27). Moreover, P-gp was significant despite the fact that it was measured in duodenal biopsies, which would be expected to contain lower and higher levels of P-gp and CYP3A4, respectively, relative to more distal regions of the intestine, where cyclosporine is primarily absorbed. Taken together, these observations indicate that, in the intestine, P-gp-mediated efflux plays a greater role than CYP3A4-mediated metabolism in determining the extent of the systemic exposure, and hence oral bioavailability, of cyclosporine. A similar conclusion was also reported for the related P-gp/CYP3A4 substrate tacrolimus from regional intestinal perfusion studies in rats (28). Thus, a dual P-gp/CYP3A4 substrate could still be subject to a significant first-pass process if absorbed in the distal region of the small intestine.

Among the four donor intestines, P-gp levels varied 2.1-fold in duodenum/proximal jejunum (Table II), consistent with results reported for duodenal pinch biopsies obtained from larger numbers of healthy volunteers (n = 8–24) (15–17). P-gp levels varied similarly or somewhat less among middle/distal jejunal (1.5- to 2.0-fold) and ileal (1.2- to 1.9-fold) regions. Within-intestine variation in P-gp expression ranged from 1.5- (HI-40) to 3.0-fold (HI-35) (Table II) and was near that reported for CYP3A4 immunoreactive protein (1.5- to 4.6-fold) (6). Despite the comparable intradonor variations in CYP3A4 and P-gp, there was no inverse correlation between the two proteins within an individual, consistent with a previous report (15). Collectively, the low interindividual variation in intestinal P-gp (≤2-fold) compared to CYP3A4 (>10-fold) (6,9), the lack of an intraindividual correlation between the two proteins and their opposing patterns of expression suggest two distinct regulatory mechanisms underlying the basal expression of P-gp and CYP3A4. Such mechanisms may or may not involve the pregnane X receptor or the vitamin D receptor, two nuclear receptors that have been shown to mediate the induction of both P-gp and CYP3A4 (29–31). Finally, these inter- and intraintestinal variations in P-gp levels, perhaps because of variations in the relevant (and as yet unknown) nuclear receptor(s), could represent additional sources for the large interindividual differences observed in the oral bioavailabilities of drugs whose extents of absorption are significantly influenced by P-gp (e.g., talinolol, cyclosporine, and tacrolimus). The current results also support the hypothesis that "absorption windows" may in part result from regional differences in intestinal P-gp (32).

For a given immunoblot, relative P-gp expression in the representative Caco-2 cell homogenate from a $1\alpha,25\text{-}(OH)_2\text{-}D_3$-treated monolayer (0.85, 1.04, and 0.91 for HI-31, -35, and -40, respectively) was within the range observed between distal jejunum and distal ileum (Table II), consistent with recent findings (21). Moreover, P-gp levels in $1\alpha,25\text{-}(OH)_2\text{-}D_3$-treated Caco-2 cell monolayers were approximately threefold higher than those in untreated monolayers (21). These results, together with the intraintestinal variation observed in the current study, imply that P-gp levels in uninduced Caco-2 cells would be more indicative of the proximal small intestine, and that P-gp expression in this widely used human intestinal cell line may not necessarily be "overexpressed," even when exposed to a P-gp inducer.

Implicit in our interpretation of the current findings is that P-gp expression correlates with function. *In vitro* kinetic studies utilizing tissue from different regions of human and rat intestine demonstrated a greater net efflux of the probe substrate etoposide in the ileal compared to the duodenal/jejunal region (1.5- and 1.8-fold for human and rat, respectively) (33). Additional rat studies revealed similar findings for talinolol (32) and digoxin (19), in which net efflux activity was ~2-fold greater in the ileal than in the jejunal region. Moreover, regional efflux activities were shown to be saturable and/or inhibitable by prototypical P-gp inhibitors (i.e., quinidine or verapamil). Although P-gp expression was not measured in corresponding tissues, the fold increases in efflux activity from proximal to distal regions were comparable to those observed for immunoreactive P-gp in the current study (Table II, Fig. 3). The *in vivo* human studies involving talinolol and cyclosporine (described above) also support a positive association between P-gp expression and function. Notably, the average proximal to distal fold increase in P-gp content observed in the current study (~2-fold) agreed well with the average percentage decrease in talinolol AUC (50%). A more rigorous study that addresses the expression–function relationship, as well as the nuclear receptors involved, may indeed become more feasible with the increasing availability of quality human intestinal tissue and the ongoing development of novel techniques (24,34).

In closing, results from the current study provide, to our knowledge, the first characterization of the expression of the *MDR1* gene product P-gp along the entire human small intestine. A better understanding of both the inter- and intraintestinal variation in this efflux transporter, and of its saturability and capacity, should further aid in the refinement of predictive *in vitro* models, as well as in the prediction of the extent of drug absorption. The regional variation in P-gp expression further supports the contention that the site of absorption could represent a significant source for the low and variable oral bioavailability of pertinent drugs, particularly those with a narrow therapeutic index.

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