Protein Expression Pattern of P-Glycoprotein Along the Gastrointestinal Tract of the Yucatan Micropig

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ABSTRACT: The purpose of this study is to characterize the distribution pattern of P-gp protein levels along the entire GI tract in the Yucatan micropig, which is being developed as a model for human drug bioavailability. Small and large intestines were freshly obtained and divided into about 37 segments and 10 segments, respectively (ca., 1 foot/segment). Epithelial cells from the small intestine were obtained by an elution method; whereas, a scraping method was applied to the large intestine. Total cellular protein was isolated from the epithelial cells. Western blot analysis using P-gp antibody showed that the amount of P-gp protein increased distally from the duodenum to the ileum over approximately a 10-fold range. P-gp protein in the large intestine was present at a higher level in the central portion, but the absolute amount was much less than what was found in the small intestine. © 2004 Wiley Periodicals, Inc. J Biochem Mol Toxicol 18:18–22, 2004; Published online in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/jbt.20001

KEYWORDS: **P-Glycoprotein; Small Intestine; Large Intestine; Enterocytes; Western Blot**

INTRODUCTION

P-gp is constitutively expressed in normal tissues such as the intestine and blood brain barrier as well as organs of elimination (kidney, liver), functioning as an efflux pump against xenobiotic substrates [1]. In the intestine, P-gp is located on the apical side of enterocytes and has been proposed to serve as a barrier to the oral absorption of foreign chemicals, including drugs [2]. Several groups have examined the distribution pattern of P-gp along the gastrointestinal (GI) tract. The conclusions drawn from these studies, however, have been inconsistent. One permeation study of the rat intestine indicated that efflux function was greater in the duodenum and jejunum relative to the ileum and colon [3]; whereas, others reported that the ileum and colon had greater efflux function [4,5]. Absorption of vinblastine from a rat intestine loop preparation suggested greatest efflux of P-gp in the jejunum [6]. Since many other physiological factors may affect drug permeability, the conclusions regarding the distribution pattern of P-gp are incomplete. Probe hybridization of *mdr*1 mRNA indicated similar levels of P-gp among the human colon, jejunum, and rectum [7]. Branched signal amplification showed that rat mRNA levels increased from the duodenum (20%), to the jejunum (36%), to the ileum (100%), and large intestine (73%) [8]. There has been no report about the protein levels of P-gp along the entire GI tract. In addition, most of the reports cited lacked details concerning which GI segments were used in the experiments; segment differences may contribute to inconsistency among studies.

Recently, pigs have received increasing attention by investigators as a potential model for pharmacokinetic and bioavailability studies. The purpose of this study was to characterize P-gp protein content of intestinal enterocytes in order to provide more direct and detailed information about the P-gp protein distribution pattern along the entire GI tract.

MATERIALS AND METHODS

Animals

Male uncastrated Yucatan micropigs (S & S Farms, Rachita, CA) were housed in runs where animals were fed twice daily (Harlan-Teklad Minipig diet 8753 containing approximately 16% protein, 3% fat, and 14% fiber) and water. Animals used in this study weighed no less than 45 kg. Ampicillin (5 mg/mL; Apothecon®, Bristol-Myers Squibb Co., Princeton, NJ) was used to maintain patency of and prevent infections in the

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vascular access port and was discontinued at least one week prior to euthanasia. Protocols were approved by the University of Arizona's Animal Care and Use Committee prior to harvesting organs.

Cell and Protein Preparation

The enterocytes of the small intestine were eluted following the protocol developed by Zhang [9]. Surface cells of the large intestine were obtained by cutting the large intestine open lengthwise, washing with saline, and scraping against the intestinal surface using a glass microscope slide with use of consistent force. Cells from intestines were homogenized and lysed for 45 min with ice-cold lysis buffer (0.1 M phosphate buffer pH 7.2, 0.1 M NaCl, 3% SDS, 2 mM DTT, 0.1 mM pepstatin A, 0.1 mM leupeptin, 1 mM PMSF). Homogenates were centrifuged at 12,000 g for 30 min at 4°C. Protein amount in the supernatant was determined by the Lowry method using the BCA protein assay reagent kit (Pierce, Rockford, IL).

Western Blot

Fifty micrograms protein from each segment was resolved on 7.5% SDS-polyacrylamide gel and electrotransferred to a nitrocellulose membrane. The membrane was incubated with the C219 anti-human monoclonal antibody (Signet, Dedham, MA), 1:1000 diluted in 1% nonfat milk in T-TBS buffer (20 μM Tris-HCl, 135 mM NaCl, 0.2% Tween-20), for about 48 h at 4°C to permit maximum blotting. The secondary antibody was a peroxidase-conjugated goat anti-mouse IgG (Zymax, San Francisco, CA), 1:1000 diluted in 1% nonfat milk in T-TBS buffer. P-gp was visualized by enhanced chemiluminescence. Band density was integrated by Scion Image (Scion Corporation, Frederick, MD). Calibration curves were established by loading 6.25, 12.5, 25, 50 μg cellular protein of segment 37 from both pigs and integrating the corresponding band density. β-actin blotting with an anti β-actin antibody (A5411, Sigma, St. Louis, MO) was used as an internal control to ensure the same protein loading amount and the consistency of protein stability during the cell and protein preparation processes. β-actin was measured sometime after P-gp determination.

RESULTS

One band with molecular weight at 169 kDa and another with 151 kDa were identified by the C219 antibody, which is consistent with the results from other investigators [10]. The P-gp protein from two pigs showed a pattern of increasing amounts moving distally from the duodenum to the ileum (Figure 1A). The 169 and 151 kDa bands were quantified together using integrated band density. The P-gp content in each segment was expressed as a percentage of total P-gp from all segments and was calculated with the calibration curves as described in Methods (Figure 1B). The magnitude of the differences among segments was shown to approximate a 10-fold range. The first several segments of the duodenum in both pigs appeared to contain no or very low levels of P-gp protein. Pig B showed a smoother increasing trend of P-gp content than pig A, which displayed a considerable decrease in segments 30–33 and segment 36 compared with the neighboring segments. Such irregular distributions were not caused by experimental errors such as protein stability, because the β-actin was shown to be at the same expression level among the segments (data not shown). Because of the limited number of animals examined, whether such irregular distribution of P-gp in certain segments is typical could not be verified. However, this did not affect the conclusion that the ileum contains a significantly higher amount of P-gp than the duodenum and jejunum. Because of the irregular shape, large lumen volume, and thin tissue, it is difficult to obtain enterocytes using the elution method with the large intestine. Therefore, the cells were gathered by a scraping method, with great care taken to avoid removing muscle cells beneath the enterocytes. The middle part of the large intestine in pig C was found to contain higher levels of P-gp protein compared with the more proximal and distal segments of the large intestine. Although there might be interindividual variation, a comparison of the P-gp protein content between the large intestine (pig C) and the small intestine (pig A) still indicated much less P-gp content in the large intestine than in the ileum (Figure 2). The absolute cell amount per segment in the large intestine, prepared by this scraping method (which was shown to collect more cells than the elution method from the small intestine from our experience) was less than that obtained in the small intestine. Thus, we conclude that P-gp protein content, either per segment or normalized by cellular proteins in the large intestine, is much less than that found in the small intestine. Interestingly, in the large intestine, there was only a single 169 kDa band of P-gp protein, compared to two bands observed in the small intestine.

DISCUSSION

The efflux efficiency of P-gp substrate by the apical enterocytes for a P-gp substrate is determined by the P-gp number or density on the cell membrane. This principle is also the basis for the application of various

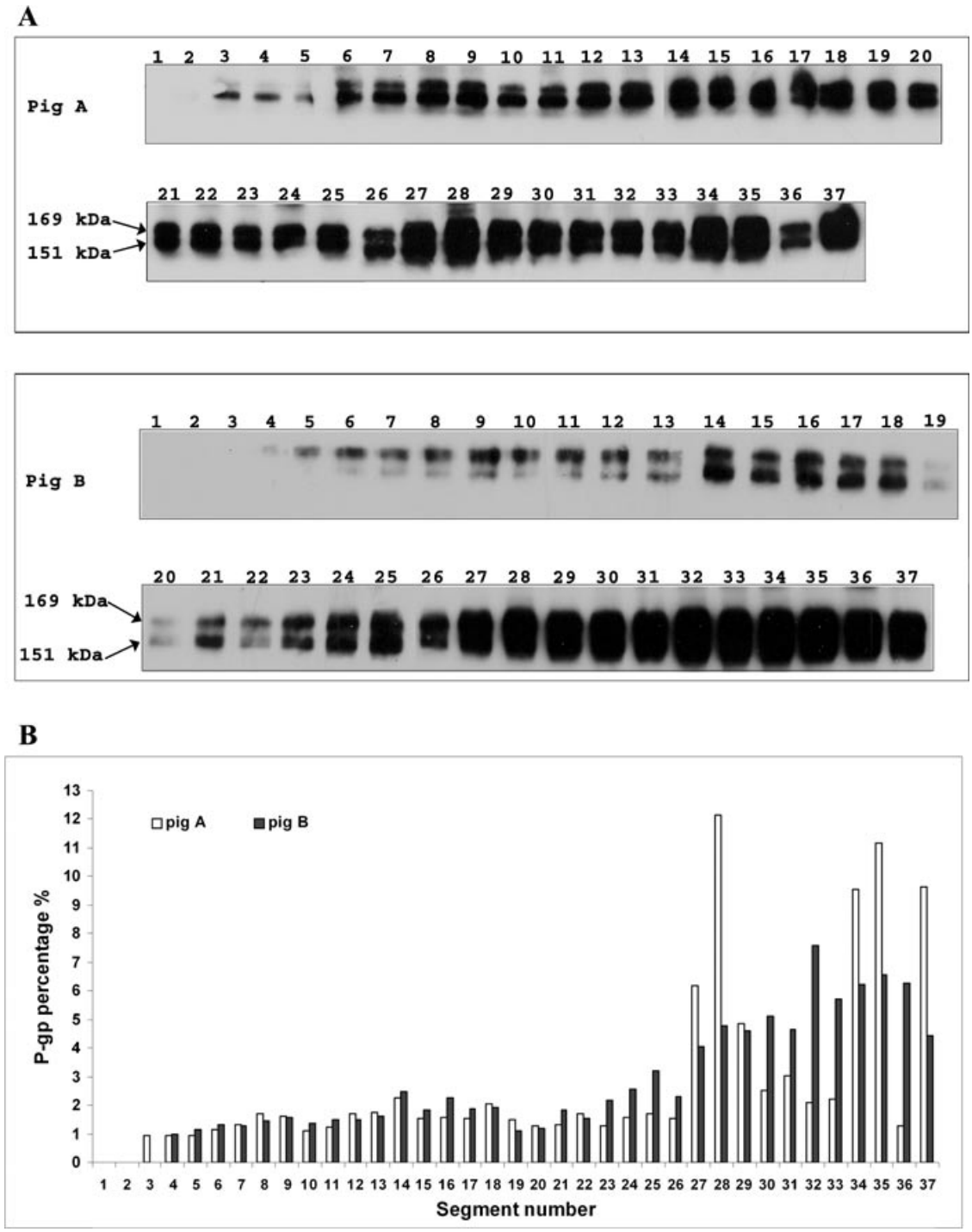

FIGURE 1. (A) Western blot of P-gp proteins along the small intestine of two pigs. The numbers from 1 to 37 indicate segments from the proximal duodenum to the distal ileum. Molecular weights are indicated by arrows. Fifty micrograms protein was used for each segment. (B) P-gp protein content in each segment was converted from band density in A and expressed as a percentage of total P-gp from all segments.

in vitro cell lines in drug absorption studies. For example, the efflux function of Caco-2, which is cultured as a monolayer, is investigated on the basis of P-gp content per cell or per cellular protein. Therefore, in the current study we compared P-gp protein content among different GI segments based on the same amount of cellular protein of the eluted enterocytes, rather than comparing the total P-gp content per segment. Elution and scraping methods are usually used to collect the GI enterocytes. The scraping method was less consistent in

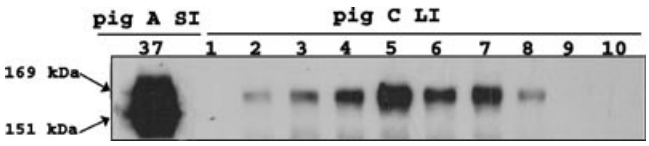

FIGURE 2. Western blot of P-gp proteins along the large intestine. Left: Fifty micrograms protein from the distal ileum segment 37 of pig A. Right: Fifty micrograms protein from different segments of the large intestine of pig C. The large intestine segment numbers from 1 to 10 represent descending order from the colon to the rectum.

terms of scraping pressure applied and cell types collected. In contrast, the elution method has been shown to be efficient and consistent in collecting enterocytes [9,11,12]. The procedure used in this study has been shown to elute villous and crypt cells successfully in the rat, but only villous cells in humans [9]. Although elution efficacy for removal of crypt cells in pigs is not known, the ultimate goal of this study would not be affected by this efficacy as long as there was consistent removal of enterocyte types from each segment.

It is widely accepted that the molecular weight of P-gp is about 170 kDa. The 151 kDa P-gp observed in this report could be explained by several possibilities; P-gp 3 isoform, degradation product, or a less glycosylated isoform. Based on DNA sequence homology, *p-gp*1 A, *p-gp*1 B, *p-gp*1 C, and *p-gp*1 D genes of the pig are comparable to human *MDR* 1 gene, and the *p-gp*3 gene of the pig is comparable to the human *MDR* 3 gene [13]. Mouley and Paine recently reported a pattern of P-gp distribution in the human small intestine similar to the pattern noted here in pigs [14]. Only *p-gp*1 A mRNA expression was found along the small intestine of the micropigs using RT-PCR; whereas *p-gp*1 B, *p-gp*1 C, and *p-gp*1 D and *p-gp* 3 mRNA expression was not observed (unpublished observation). This evidence suggested that the 151 kDa band is less likely to be the P-gp 3 isoform. Denaturation of protein samples either at room temperature for 2 h or at 90°C for 5 min showed the same results, indicating the 151 kDa isoform is not due to heating degradation (data not shown). To check whether there was degradation during cell elution, which lasted about 3 h, surface cells of several segments were removed promptly by the scraping method following sacrifice of the pigs. Western blotting results of scraped cells also showed the same 169 and 151 kDa bands as those seen from eluted cells, further indicating the 151 kDa isoform was not a degradation product (data not shown). Thus, a less glycosylated isoform is suggested for the 151 kDa isoform, which had a similar protein level to the 169 kDa isoform across the entire small intestine (Figure 1). It is unclear why the large intestine only contains one form of P-gp. One possibility is that the regeneration rate of surface cells in the small intestine, being faster than that in the large intestine, resulted in a significant amount of an immature isoform, for example, a less glycosylated isoform.

In conclusion, the content of P-gp, which varied about a 10-fold range, was found to increase from the proximal duodenum to the distal ileum. The large intestine contained less P-gp than the jejunum and ileum. Within the large intestine, P-gp content was higher in the middle segments than in the distal and proximal segments. Compared to mRNA measurements and permeation studies, we provide more direct evidence and more detailed information about the distribution pattern of P-gp protein. Such information would be helpful for functional studies of P-gp and the better understanding of the role of P-gp in the GI tract. For example, why should the ileum have the highest level of P-gp while the jejunum acts as the major absorption site along the entire GI tract and should, therefore, contain higher levels of P-gp? One possible explanation is that the concentration of P-gp substrates increases distally due to substrates being extruded back into the lumen and accumulated distally, as well as water being efficiently removed from the ileum.

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