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Multidrug resistance gene (MDR-1) expression in the colonic mucosa of patients with refractory ulcerative colitis

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Summary

Background. P-Glycoprotein (P-gp), a product of the MDR-1 gene, is a transmembrane efflux pump involved in drug transport, first described in cancer refractoriness. In the normal bowel, P-gp is detectable on superficial epithelial cells, but has not been described in crypt epithelium. The role of P-gp and its intestinal expression in steroid-refractory ulcerative colitis (UC) are controversial. Aim. To compare P-gp immunostaining pattern in colonic epithelial cells of steroid-refractory versus steroid-responder UC patients. Methods. P-gp was assessed by immunohistochemistry in rectal biopsies obtained from 19 patients with active UC, including pre-surgical samples from 11 refractory patients who underwent colectomy, and 8 responders. We devised a 5-point (0-4) score, according to the percentage of epithelial surface with positive immunostaining in the superficial and crypt epithelium (apical, lateral and cytoplasmic areas). Results. Compared with responders, steroid-refractory patients had significantly higher immunostaining scores in the superficial epithelium, both in apical (2.8±0.5 versus 1.1±0.5, p=0.023) and cytoplasmic cellular areas (2.7±0.5 versus 1.2±0.5, p=0.032). Positive immunostaining of the superficial epithelium was frequently detected in refractory patients (apical: 9/11 cases, cytoplasmic:10/11 cases) but was only observed in 4/8 responders. P-gp was also detected in similar areas of the crypt epithelium in 6/11 refractory patients, while it was infrequent in the group of 8 responders (1 apical case, cytoplasmic 2 cases). Samples from the mucosa of normal ileal pouch-anal anastomoses obtained several years after the surgical procedure had a P-gp immunostaining pattern which was similar to that of rectal samples from patients with refractory UC. Conclusions. These results suggest a critical role of P-gp overexpression in steroid-refractory UC.

Index (palabras claves): Ulcerative-colitis, Steroid-resistance, Inflammatory-Bowel-Diseases, MDR1, P-Glycoprotein

Resumen

Expresión del gen de multirresistencia a drogas (MDR-1) en la mucosa colónica de pacientes con colitis ulcerosa refractaria

Antecedentes. La glicoproteína P (P-gp), un producto del gen MDR-1, es una bomba de eflujo transmembrana involucrada en el transporte de drogas, descripta por primera vez en el cáncer refractario. En el intestino normal, P-gp se detecta sobre las células epiteliales superficiales, pero no se la ha descrito en el epitelio de las criptas. El papel de P-gp y su expresión intestinal en la colitis ulcerosa (CU) refractaria a esteroides es controvertido. Objetivo. Comparar el patrón de inmunotinción de P-gp en células epiteliales colónicas de pacientes con CU refractaria vs.
respondedora a esteroides. **Métodos.** Se estudió P-gp por inmunohistoquímica en biopsias rectales obtenidas de 19 pacientes con CU activa, incluyendo muestras prequirúrgicas de 11 pacientes refractarios que fueron sometidos a una colectomía y muestras de 8 respondedores. Ideamos un score de 5 puntos (0-4), según el porcentaje de superficie epitelial con inmunotinción positiva en el epitelio superficial y criptico (áreas apical, lateral y citoplásmica). **Resultados.** Comparados con los respondedores, los pacientes refractarios a esteroides tenían scores de inmunotinción significativamente mayores en el epitelio superficial, tanto en las áreas celulares apical (2.8±0.5 vs. 1.1±0.5, p=0.023) como citoplásmica (2.7±0.5 vs. 1.2±0.5, p=0.032). Se detectó frecuentemente inmunotinción positiva en el epitelio superficial en los pacientes refractarios (apical: 9/11 casos, citoplásmica: 10/11 casos), pero la misma se observó sólo en 4/8 respondedores. P-gp también se detectó en áreas similares del epitelio de las criptas en 6/11 pacientes refractarios, en tanto que fue infrecuente en el grupo de los 8 respondedores (1 caso en el área apical y 2 en la citoplásmica). Fueron estudiadas biopsias de la mucosa de la anastomosis pouch ileal - anal, obtenidas varios años después del procedimiento quirúrgico, observándose un patrón de inmunotinción de P-gp similar al de las muestras rectales de los pacientes con CU refractaria. **Conclusión.** Estos resultados sugieren un papel crítico de la sobre-expresión de P-gp en la CU refractaria a esteroides.

The multidrug resistance gene MDR-1 encodes a 170 kDa glycosylated transmembrane protein, P-glycoprotein (P-gp), which belongs to the ATP binding cassette superfamily of transport proteins. P-gp is found at variable expression levels in diverse cellular types, including lymphocyte subsets, macrophages, mononuclear cells and several tissues with barrier function, such as intestinal epithelial cells. P-gp is an efflux pump, which acts as a unidirectional ATP-dependent transport system (inside-to-outside) for structurally diverse amphiphilic and hydrophobic compounds. P-gp plays a role in the pharmacokinetics of several drugs and additionally, may exhibit other relevant functions, still under research, such as intestinal homeostasis and the pathogenesis of inflammatory diseases. Overexpression of P-gp was first described in multidrug resistant tumor cells and was implicated in cancer chemotherapy refractoriness. In such context, it was shown that P-gp pumps anticancer drugs out of cells, thus reducing the efficacy of treatment.

Since some therapeutic agents for inflammatory bowel disease (IBD) are also P-gp substrates, it has been hypothesized that P-gp dependent drug pharmacokinetics plays an important role in the refractoriness of ulcerative colitis (UC). However, to date there are few studies published about MDR-1 gene expression in inflammatory bowel disease and findings are controversial. MDR-1 gene expression has been described to correlate with genetic polymorphisms, perhaps reflecting a constitutive condition. Recent studies have suggested an association between MDR-1 gene polymorphisms and ulcerative colitis. Moreover, the coincidental location of the MDR-1 gene on chromosome 7q21.1 with that of an IBD susceptibility site found in genome wide screens, seems to suggest that MDR-1 is a candidate gene, which may play a role in the pathogenesis of ulcerative colitis or in its response to treatment.

The MDR-1 gene has been suggested to present differential pattern expression or functional activities, depending on the cellular type expressing P-gp, and we hypothesize that it can also occur in different epithelial cell types such as in superficial and crypt epithelia. Therefore, an immunohistochemical method to identify and compare P-gp immunostaining pattern by areas (superficial versus crypt epithelium) and sub-cellular location (apical, lateral and cytoplasmic immunostaining) in colonic biopsies, could be a useful tool to assess pathological conditions.

In this study, our aim was to investigate whether the immunostaining pattern of P-gp in colonic biopsies of patients with steroid-refractory ulcerative colitis differs from that of patients with steroid-responsive disease. In addition, to determine a potential constitutive overexpression of P-gp in patients with steroid-refractory ulcerative colitis, we also aimed to assess MDR-1 gene expression in the distal small bowel mucosa, a high P-gp expressing site, from normal ileal pouch-anal anastomoses (IPAA) of patients who had undergone surgery for steroid-refractory UC. We speculate that these data could provide information about the constitutive P-gp expression, since IPAA is a condition free of
treatment and inflammation, factors which may modify the results.

**Material and Methods**

Immunohistochemical P-gp expression was studied in formalin-fixed, paraffin-embedded rectal biopsy samples obtained by colonoscopy from 19 patients with UC who had been hospitalized several years before due to severely active disease and treated with high doses of intravenous steroids (hydrocortisone, 400 mg during 7 days). According to their response, patients were grouped in steroid-refractory (11 patients) and steroid-responders (8 patients).

The diagnosis of UC had been previously confirmed at our Center based on standard criteria including clinical, radiological, endoscopic and histological parameters. Disease activity was assessed based on a modified clinical-endoscopic disease activity index (DAI), which includes clinical features (stool frequency and rectal bleeding), colonoscopic findings and the physician’s rating of disease activity. Endoscopic assessment was performed by colonoscopy using Carbonnel’s criteria for moderate and severe colitis. Physician’s assessment of disease severity used in the present study consisted of the combined interpretation of the patient's general sense of well-being, performance status and biological signs of severe disease activity (fever, tachycardia, anemia, accelerated erythrocyte sedimentation rate and low serum albumin). A DAI score ≥ 9 was required for the disease to be classified as severe.

Steroid responsiveness was defined as a decrease in the disease activity index equal to or greater than 3 points after the 7th day of treatment, with achievement of a final score ≤ 2 on the 8th week.

Steroid refractoriness was defined as clinical worsening or no improvement or a decrease in DAI < 3 points after the 7th day of treatment.

Patients with steroid-refractory ulcerative colitis were referred to urgent surgery. Responder patients were followed with 3 grams of sulphasalazine and decreasing doses of oral prednisone, which was discontinued at week 8, after disease activity assessment and biopsy collection.

The P-gp immunohistochemical study was performed in the rectal pre-surgical biopsy samples obtained from the group of eleven steroid-refractory patients who underwent colectomy and IPAA, and in the biopsies obtained from the eight patients who were responders at week 8. The immunostaining patterns between both groups were compared. All biopsies were obtained according to the usual diagnostic and follow-up protocol for the management of UC used at our Center. This study was approved by the Institutional Review Board and Ethics Committee of the Bonorino Udaondo Gastroenterology Hospital, Buenos Aires, and informed consent was obtained from all patients.

Additionally, P-gp immunostaining study was carried-out in endoscopic pouch biopsies obtained from those five steroid-refractory previously colectomized patients, who had a normal IPAA after long term follow-up. These biopsies were taken several years later to undergone ileostomy closure (mean time: 7 years, range 5 to 16 years) and all five patients were free of anti-inflammatory or antibiotic treatment (aminosalicylates, antibiotics, steroids, or immunosuppressants). A normal pouch was defined by a Pouchitis Disease Activity Index (PDAI) <7 points.

Samples were immunostained with the avidin-biotin-peroxidase method, using a monoclonal antibody (JSB-1-Novocastra) directed against P-Glycoprotein. P-gp immunostaining patterns were assessed with light microscopy by two experienced pathologists who were blinded to the patients' status, and the Mann-Whitney test was used to compare the scores between groups (means±SEM).

Superficial epithelial cells and crypt epithelial cells were separately analyzed in both areas and categorized as apical (brush border membrane), lateral and cytoplasmic locations.

A positive epithelial reaction was observed as a brown staining signal, located in the apical brush border membrane, lateral membrane and cytoplasmic areas.

According to the percentage of epithelial surface positively immunostained on superficial and crypt epithelium per area, a 5-point (0-4) score scale was devised as follows:

0. Negative immunostaining
1. Scarce and weakly positive immunostaining, involving 25% of the epithelial surface.
2. Focal positive immunostaining, involving >25% to 50% of the epithelial surface.
3. Focal positive immunostaining, involving >50% to 75% of the epithelial surface.
4. Strong and diffuse positive immunostaining >75% of the epithelial surface.

(Figures 1a and 1b)
Results
A higher P-gp expression was observed in colonic biopsies from patients with steroid-refractory UC, compared to the samples of responder cases. (figure 2)
The percentage of epithelial surface expressing P-gp showed higher scores in patients with refractory UC than in responders, in all investigated areas. Statistically significant differences were observed in both apical and cytoplasmic areas of the superficial epithelium. (figure 3a)
In the refractory group, 9 of 11 patients had positive P-gp expression in the superficial epithelium of the apical area and 10 patients in the cytoplasmic area. In the responder group, 4 of 8 patients had P-gp expression in both the apical and cytoplasmic areas. Of interest, in the crypt epithelial area, in contrast to what is reported in normal conditions, P-gp expression was also frequently detected. In the refractory group, 6 of 11 patients, had P-gp expression in both the apical and cytoplasmic areas, and in the responder group, only 1 of 8 patients had positive P-gp expression in the apical area and 2 patients in the cytoplasmic area. The absence of significant differences could be secondary to the small number of patients studied. Finally, all patients expressed some degree of P-gp staining in the lateral area of the superficial epithelium and crypts. (figure 3b)
We also showed a moderate correlation of P-gp expression with disease activity in the apical area and the cytoplasmic region (r=0.61, p=0.00524 and

Figures 1a and 1b. P-gp immunostaining of formalin-fixed, paraffin-embedded sections from endoscopic biopsies of patients with UC. A positive epithelial reaction was observed as a brown staining signal, detected either as enhanced diffuse, focal, or scarce weakly-stained patterns located in the apical brush border membrane (A), lateral (L) and cytoplasmatic (C) areas. Superficial epithelium (Fig. 1a) and crypt epithelium (Fig. 1b) were separately evaluated. Arrows indicate the P-gp location and score values. (40X).
Figure 2. P-gp immunostaining in rectal biopsies obtained from patients with steroid-refractory UC (panel a) compared with steroid-responsive UC patients (panel b). (25X).

![Figure 2](image1)

**Figure 3a.** Bar graphs depicting P-gp immunostaining epithelial scores (mean±SEM), which show higher values in steroid-refractory UC patients versus responder cases, in all investigated areas. There were statistically significant differences between the apical and cytoplasmic areas of the superficial epithelium.

![Figure 3a](image2)

**Figure 3b.** Frequencies of positive immunostaining observed on superficial and crypt areas, categorized by type of sub-cellular location (apical, lateral or cytoplasmic) in both groups.

![Figure 3b](image3)
The analysis of the normal IPAA mucosa obtained several years after surgery in the 5 patients with refractory UC, showed no significant difference in the mean P-gp scores when compared to colonic biopsies from the group of 11 patients with refractory UC. (Table 1) P-gp immunostaining in pouch mucosa is illustrated in figure 4.

### Table 1. Mean scores of the superficial epithelium of both refractory UC and normal pouch show similar patterns.

<table>
<thead>
<tr>
<th>Superficial Epithelium</th>
<th>Normal Pouch N=5</th>
<th>Refractory UC N=11</th>
<th>P (Mann-Whitney)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apical</td>
<td>2.40±0.81</td>
<td>2.81±0.46</td>
<td>NS</td>
</tr>
<tr>
<td>Lateral</td>
<td>2.60±0.51</td>
<td>2.90±0.41</td>
<td>NS</td>
</tr>
<tr>
<td>Citoplasmatic</td>
<td>2.00±0.71</td>
<td>2.72±0.45</td>
<td>NS</td>
</tr>
</tbody>
</table>

**Figure 4. Image of epithelial P-gp distribution in a normal ileal pouch-anal anastomosis.**

**Discussion**

In this study we found that in steroid-refractory UC, the distribution of P-gp expression was significantly more diffuse in superficial epithelial cells, and there were more cases with positive expression, when compared to the responder group of patients.

In patients with refractory UC, we also observed a frequent and diffuse pattern expression in the crypt cell’s area, while under normal conditions P-gp was reported as undetectable.8,13-14

Additionally, it is worth noting that patients with refractory UC showed similar P-gp expression patterns prior to surgery (in rectum) and post surgery (in the long-term normal IPAA, in condition free of treatment and inflammation).

P-gp was initially identified by its ability to confer multidrug resistance in mammalian tumor cells and implicated in refractoriness to cancer chemotherapy, resulting from the P-pg-mediated efflux of drug from tumor cells.15-23

In addition to its function as transporter of certain chemotherapeutic agents, P-gp is also a transmembrane ATP-binding transporter of several lipophilic and amphipathic molecules.24 Several possible mechanisms of action are still under discussion.46-47

The tissue-specific expression of the MDR-1 gene has been investigated in humans, mice, rats, and hamsters. In humans, the P-glycoprotein is located...
on the surface of the bile ducts of hepatocytes, apical surface of proximal tubules in kidneys, columnar epithelial cells of the intestine, and capillary endothelial cells of the brain and testes.  

Immunohistological studies performed in human small intestine have reported that P-gp is mainly expressed in the apical surfaces of superficial epithelial cells of the distal small bowel and colon, but not in crypt epithelium.  

Based on P-gp location in the intestinal epithelium and results of several experimental studies, it has been assumed that intestinal P-gp may play a role in limiting the absorption of orally administered drugs by extruding them from the epithelial cells into the intestinal lumen. The observation that P-gp inhibitors may modify the pharmacokinetics of certain drugs provides additional support to this hypothesis. Hsing et al have demonstrated that the release of P-gp probes [3H] daunomycin from brush-border membrane vesicles and rhodamine 123 from rat everted small intestine were inhibited by P-glycoprotein modulators: diltiazem, colchicine, and verapamil. It was also shown that the efflux of etoposide, an anticancer drug, from rat everted small intestine was inhibited by the addition of C219, a monoclonal anti-P-glycoprotein antibody. However, it is currently assumed that besides a role limiting drug absorption, secretion (translocation from the blood side to apical side) via P-glycoprotein into gut lumen might play an important role in pharmacokinetics and pharmacodynamics of corticosteroids and other P-gp substrates.

Nevertheless, issues such as the magnitude, characteristics, clinical relevance and assessment of these mechanisms require further clarification. Variations in MDR-1 gene expression and/or genotypes have been considered as factors able to modify the pharmacokinetics of a wide range of drugs to influence steroid requirements in rheumatoid arthritis and systemic lupus erythematosus, to induce cyclosporin refractoriness in graft rejection, and also implicated in drug-resistance in epilepsy.  

There is also additional evidence suggesting a role of MDR-1 gene in IBD drug pharmacokinetics. “In vitro” studies have shown that high MDR-1 expressing-cells actively transport diverse drugs used for inflammatory bowel disease treatment, such as glucocorticosteroids, cyclosporin, methotrexate. Additionally, MDR specific inhibitors may increase intracellular cortisol and cyclosporin.  

In spite of these findings, the literature regarding MDR-1 gene expression and/or its function in inflammatory bowel disease is scarce, and these relevant issues require further investigation. Farrell et al have reported increased P-gp expression in peripheral blood lymphocytes (PBL) and intestinal epithelial lymphocytes in refractory IBD, and have hypothesized a critical role of MDR1, by pumping steroids out of target cells. They also detected high PBL P-gp levels in treatment-free patients with normal long-term ileoanal pouches (IPAA), who had undergone surgery due to refractory UC, suggesting that this finding involved a constitutive condition.

However, controverted issues were highlighted by different authors. As an example, Ho et al suggest that Farrel’s study could not show significant differences between PBL MDR-1 gene expression in refractory colectomized and active nonsurgical UC groups. Another result in Farrel’s study is that intestinal epithelial cells exhibited lower P-gp expression than PBL, as well as only moderate correlation with lymphocyte levels. The authors have suggested that this finding was a technical consequence of flow cytometry, because the technique uses different conjugates to label epithelial cells (fluorescein isothiocyanate-FITC) and lymphocytes (phycoerythrin).  

We hypothesized that the results of P-gp global expression from pooled epithelium could be affected by physiological variations according to the location (superficial epithelium versus crypt).

Lymphocyte MDR-1 expression has been reported to depend on different subsets of lymphocytes, their activation and also the clinical status (inflammatory bowel disease type or healthy individual).  

Additionally, MDRI seems to exhibit different functional activities depending on the cellular site of expression (as has been shown in irradiated mdr1a knockout mice after bone marrow reconstitution) and it could be similar for different epithelial cell types.

Our study suggests that the comparison of immunohistochemical staining patterns by areas and subcellular location may be a satisfactory method to detect differences in the response of UC to treatment. We have shown equivalent expression of P-gp in active UC under therapy, and long-term IPAA with no treatment or inflammation. Although the organ investigated is different, the findings suggest that it may reflect a predisposition to refractoriness, beyond colon damage and drug-influences.
Hence, we hypothesize that the significance of P-gp expression in the IPAA mucosa seems to be equivalent to PBL over-expression described by Farrell et al., thus supporting a constitutive condition of refractoriness.

In conclusion, our data support the hypothesis that over-expression of P-gp in superficial and crypt epithelial intestinal cells may play a critical role in steroid refractoriness of UC.

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