Expression and functional analysis of intestinal organic cation/L-carnitine transporter (OCTN) in Crohn's Disease

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Abstract

Background: The IBD5 locus is a genetic risk factor for IBD, particularly Crohn's Disease, coding for the organic cation/carnitine transporters (OCTN1 and 2). Two variants of OCTN are associated with susceptibility to Crohn's Disease. Modified transport of carnitine in vitro has been reported for a polymorphism of OCTN1. The aim was to investigate the function of intestinal OCTNs in IBD in relation to genetic polymorphisms.

Methods: Intestinal tissue was obtained from endoscopic biopsies and surgical resections from IBD patients (n = 33 and 14, resp.) and controls (n = 22 and 14, resp.). OCTN protein levels were measured in intestinal biopsies and carnitine transport was quantified in intestinal resections.

Results: OCTN1 protein levels were significantly higher in ileal versus colonic tissue (2.95% ± 0.4 vs 0.66% ± 0.2, resp.; p < 0.0002). OCTN1 expression was higher in Crohn's disease patients with mutant homozygous or heterozygous genotypes (0.6% ± 0.1 vs 3% ± 0.8, resp., p < 0.02). Carnitine
transport was very rapid and Na+ dependent (10 s). It was not different comparing Crohn’s Disease and control groups (0.45 ± 0.12 vs 0.51 ± 0.12 μM carnitine/mg prot/min, resp.). Carnitine transport tended to be higher in subjects with mutant homozygous and heterozygous OCTN1 and OCTN2 genotypes (0.19 vs 0.59 and 0.25 vs 0.6, respectively).

Conclusions: The present data reveal that OCTN protein levels appear to be similar in intestinal tissue from Crohn’s Disease patients and controls. Overall, ileal carnitine transport appears to be equal in Crohn’s Disease and control groups. However, there was a trend towards higher carnitine transport in subjects with OCTN1 and OCTN2 mutations.

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1. Introduction

Crohn’s Disease (CD) is a chronic inflammatory bowel disorder (IBD) whose multifactorial pathogenesis is incompletely understood. The currently held hypothesis implicates aberrant immune response to the intestinal microbiome, genetic predisposition and environmental factors such as microbes, diet or tobacco use. The importance of genetic factors in the pathogenesis of IBD is now well established, particularly for CD. First-degree relatives of CD patients have a relative risk to develop the disease of up to 35. A recent genome wide meta-analysis identified 71 genetic loci conferring susceptibility to the development of CD.

The IBD5 locus on the 5th chromosome was identified as conferring a 2 to 6 fold risk to develop CD. This region codes for two genes (SLC22A4 and SLC22A5) encoding the organic cation/carnitine transporters (OCTN) 1 and 2. Moreover, OCTN variants may interact with NOD2 to magnify the risk for CD. The association between risk for CD and the IBD5 locus has been replicated several times. A study by Silverberg et al. confirmed the association with SLC22A4 polymorphism. Peltekova et al. reported two variants in the OCTN cluster (1672C/T for OCTN1 and −207G/C for OCTN2) which form a haplotype associated with susceptibility to CD. Using fibroblasts in which the mutated OCTN1 gene was transected, the same group observed modified function of the transporter, with a reduced amount of carnitine transported in vitro.

Carnitine is an essential nutrient, acting as a cofactor for the beta-oxidation of long chain fatty acids in mitochondria. The OCTN transporters mediate its intestinal uptake, as well as that of several other important organic cations, including drugs (such as beta-lactam antibiotics) and xenobiotics. Butyrate transported as butyrylcarnitine via OCTN and propionylcarnitine have important pleiotropic effects in the gut. The colonic epithelium utilizes short-chain fatty acids, particularly butyrate, as its preferred energy source. Butyrate administration by enemas has been reported to decrease colonic inflammation in IBD. Tamai et al. originally identified the organic cation and carnitine transporter genes. This gene family comprises three members: OCTN1, 2 and 3, each of which are expressed in various tissues, including the small bowel and colon. Although OCTN1 and OCTN2 genes have a similar structure, their functional characteristics are distinct.

Table 1 Characteristics of patient groups.

<table>
<thead>
<tr>
<th>Colonoscopic biopsy group (n=55)</th>
<th>CD (26)</th>
<th>UC (7)</th>
<th>C (22)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male n (%)</td>
<td>17 (65)</td>
<td>3 (43)</td>
<td>12 (55)</td>
<td>0.008</td>
</tr>
<tr>
<td>Female n (%)</td>
<td>9 (35)</td>
<td>4 (57)</td>
<td>10 (45)</td>
<td>0.008</td>
</tr>
<tr>
<td>Mean age (SD)</td>
<td>25 (16.3)</td>
<td>46 (12.6)</td>
<td>44 (16.8)</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Ileal resection group (n=28)</th>
<th>CD (10)</th>
<th>UC (4)</th>
<th>C (14)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male n (%)</td>
<td>5 (50)</td>
<td>2 (50)</td>
<td>8 (57)</td>
<td>ns</td>
</tr>
<tr>
<td>Female n (%)</td>
<td>5 (50)</td>
<td>2 (50)</td>
<td>6 (43)</td>
<td>ns</td>
</tr>
<tr>
<td>Mean age (SD)</td>
<td>35 (13)</td>
<td>37 (6.1)</td>
<td>59 (15.9)</td>
<td>0.0007</td>
</tr>
</tbody>
</table>

Diagnoses among the control group

<table>
<thead>
<tr>
<th>Biopsy group</th>
<th>Resection group</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRC screening n (%)</td>
<td>12 (55)</td>
</tr>
<tr>
<td>CRC n (%)</td>
<td>1 (4)</td>
</tr>
<tr>
<td>IBS n (%)</td>
<td>7 (32)</td>
</tr>
<tr>
<td>Diverticulitis n (%)</td>
<td>2 (9)</td>
</tr>
</tbody>
</table>

CRC = colorectal cancer; CD = Crohn’s disease; UC = ulcerative colitis; and C = controls.
The OCTN1 polypeptide is a multispecific and bidirectional organic cation and carnitine transporter, sodium and pH dependent. It has a lower affinity for carnitine than OCTN2.27 The expression and intestinal localization of these transporters were first demonstrated using human intestinal epithelial cells.28 The predominant role of apical OCTN 2 was subsequently confirmed using the OCTN2−/− knock out mouse model.29

Despite genetic studies suggesting that polymorphisms in OCTN transporters lead to increased susceptibility to CD, no study has investigated the functional transport of carnitine by OCTNs directly in intestinal tissue in IBD. The aims of this study were therefore to quantify intestinal tissue levels of OCTN proteins in intestinal tissue from CD and control patients, as well as to compare carnitine transport activity of OCTNs, and to correlate these results with genotypic analysis of polymorphisms.

2. Materials and methods

2.1. Patients and tissues

Patients were recruited from three hospitals of the McGill University Health Center (MUHC) in Montreal (2 adult and 1 pediatric). Approval of the protocol was obtained from the MUHC Ethics Committee and all patients gave informed consent. Patients recruited were scheduled to undergo a colonoscopy or an ileocolonic resection. Two kinds of tissue specimen were collected: i) colonoscopic biopsies (6 ileal and/or colonic specimens) from macroscopically non-denuded and non-ulcerated areas; or ii) a non inflamed ileal segment (2×2 cm) at the time of surgical resection. Tissue specimens were transported to our IBD lab immediately. Before freezing (−80 °C) the ileal resection, the mucosa was scraped using a glass microscope slide.

2.2. Western blot analysis

The protocol used was modified from Lahjouji et al.30 Biopsies were homogenized (Ultra Turrax T25 polytron, Imlab, France) 1 min at 11,000 rpm in 350 μl of an ice cold buffer. After addition of denaturized buffer (4×) containing 8% sodium dodecyl sulfate (SDS), 40% glycerol, 0.25 M Tris HCl and 0.0001% bromophenol blue, the samples were boiled (5 min). Similar amounts of protein were run on 10% SDS-polyacrylamide gel electrophoresis and transferred to a PVDF membrane (Millipore Corporation, Billerica, MA). Goat anti human OCTN1 and OCTN2 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were used as primary antibodies overnight at 4 °C, at dilutions of 1/100 and 1/200 respectively. Donkey anti goat conjugated to horseradish peroxydase (Santa Cruz Biotechnology) was used as secondary antibody. The membranes were developed with Immobilon Western® reactant (Millipore) and read with a Luminescent Image Analyser (LAS 4000, Fuji, Tokyo, Japan). β-Actin (Santa Cruz Biotechnology) was used as an internal standard. Mouse kidney tissue was used as a positive control.

2.3. Brush border membrane vesicle isolation

Brush border membrane (BBM) vesicles were prepared from frozen ileal specimens using a protocol modified from the techniques described by Shirazi-Beechey et al.31,32 All steps were performed at 4 °C. Intestinal mucosal specimens were homogenized (1 min at 16,000 rpm) in a volume of buffer 50 times the weight containing mannitol and HEPES Tris (50 and 2 mM, respectively, pH 7.0). After addition of MgCl2 (10 mM), the homogenate was centrifuged (2000 g×15 min) using an Allegra 6R centrifuge (Beckman Coulter, Fullerton, CA). The supernatant was then centrifuged

| Table 2 | OCTN genotyping results. |
|---|---|---|---|
| OCTN1 | |  |
| n (%) | Wild type (CC) | Heterozygote (TC) | Homozygote (TT) |
| C n (%) | 18 (31.6) | 26 (45.6) | 13 (22.8) |
| CD n (%) | 9 (31) | 12 (41.4) | 8 (27.6) |
| UC n (%) | 3 (37.5) | 5 (62.5) | 0 (0) |
| OCTN2 | |  |
| n (%) | Wild type (GG) | Heterozygote (CG) | Homozygote (CC) |
| C n (%) | 16 (39) | 17 (41.5) | 8 (19.5) |
| CD n (%) | 8 (40) | 8 (40) | 4 (20) |
| UC n (%) | 2 (28.6) | 4 (57.1) | 1 (14.3) |

C = controls; CD = Crohn’s disease; and UC = ulcerative colitis.
(27,000 g × 30 min) using a Sorvall R C2-B centrifuge. The pellet was resuspended in 3 ml of a suspension buffer (pH 7.5) containing mannitol (125 mM), MgSO₄ (0.1 mM), KCl (250 mM) and HEPES Tris (50 mM), using a syringe and a 26-gage needle. The ensuing solution was again centrifuged (43,000 g × 20 min). The final pellet was suspended in 200 μl of the suspension buffer. BBM were stored in liquid nitrogen until use. BBM purity was assessed by measuring enrichment of alkaline phosphatase. 

2.4. Carnitine uptake

The method employed was modified from Lahjouji et al., Stevens et al. and Li et al. 

Uptake of [3H]carnitine (83 Ci/mmole, Amersham Biosciences, Piscataway, NJ) was measured using a filtration method with manifold and nitrocellulose filters of 0.45 μm pore size (Millipore Corporation). Aliquots of 200 μg of BBM were used for each experiment. The filters were then wetted with cold suspension buffer. BBM were removed from the liquid nitrogen for 20 s at room temperature. They were then mixed during the predetermined time of the reaction with 100 μl of transport buffer (pH 7.5) containing 2 μCi [3H]carnitine (10 nM), 1 mM L-carnitine (ICN Biomedicals (Aurora, OH)), 125 mM mannitol, 0.1 mM MgSO₄, 250 mM KCl, 150 mM NaCl and 50 mM HEPES Tris. The reaction was stopped by the addition of 2 ml of cold suspension buffer. BBM were then filtered on the manifold with another 2 ml of cold suspension buffer. The filters were recovered and dried. 6 ml of Ecolite solution (ICN, Irvine, CA) was added and the 3H radioactivity determined using a Beckman Coulter LS 5801 scintillation counter (Fullerton, CA).

2.5. DNA extraction and genotyping

DNA was extracted from 3 ml whole blood or from frozen intestinal tissue with the Purgene DNA purification kit (Gentra, Minneapolis, MN). The genotyping of the common OCTN polymorphisms was performed using primer extension chemistry and mass spectrometric analysis (iPlex assay, Sequenom, San Diego, CA) on the Sequenom MassArray at the Montreal Heart Institute of the Université de Montréal. The following SNP were used: OCTN1 (SLC22A4) 1672C-T (rs1050152), OCTN2 (SLC22A5)-207G-C (rs2631367). The first is a C-T substitution in SLC22A4 that cause an amino acid substitution L503F. The second is a G-C transversion in the SLC22A5 promoter.
2.6. Statistical analysis

Statistical comparisons and analyses of data for continuous variables were performed using ANOVA and post hoc Tukey test or non-paired Student-t test. For qualitative data, the Fisher exact test was used. Differences between means were considered significant with p < 0.05.

3. Results

3.1. Characteristics of patients

Among the 86 subjects recruited, 4 did not give written consent to participate, leaving 82 subjects. Ileal and colonic endoscopic biopsies were obtained by colonoscopy in 55 patients, while ileal surgical resections were available in 28 patients (one patient provided both biopsies and ileal resection). Subjects were classified into three categories according to their diagnosis: CD, UC and controls based on standard clinical, endoscopic, radiologic and histopathological criteria. The characteristics of patients by diagnosis for both the colonoscopic biopsy and ileal resection groups are shown in Table 1. Among the former group, control patients were scheduled to undergo a colonoscopy to screen for tumors or for other, non-IBD indications (Table 1). The indications for resection are shown in Table 1 as well.

There were more males having CD in the colonoscopic biopsy group. The mean age of CD patients undergoing colonoscopy was significantly lower than in the UC and control groups. These latter subjects were mostly undergoing screening for colorectal cancer (CRC). As would be expected, the mean age of CD patients undergoing an ileal resection was higher than that in CD group undergoing a colonoscopy, given that surgery in CD is frequently delayed. Mean age of controls patients in the ileal resection group was significantly higher because CRC occurs at a more advanced age.

3.2. OCTN genotype analysis

The genotyping results are summarized in Table 2. Unfortunately, blood samples were not available for all patients,
which did not allow for complete genotype assessment. For the OCTN1 gene, the mutant homozygous 1672 TT genotype was found in 22.8% of all patients. There was no difference in the prevalence of wild type, mutant heterozygous or homozygous genotype in the control or CD group (p = 0.79). No mutant homozygous TT genotype was found in the UC group. For the OCTN2 gene, the mutant homozygous −207 CC genotype was found in 19.5% of all patients. Once again, the frequency of wild type, mutant heterozygous and homozygous genotypes at this locus was similar in the CD and control groups (p = 0.8). For both OCTN1 and OCTN2 more mutant heterozygous and fewer homozygous genotypes were found in UC than in CD and control patients (Table 2).

3.3. Intestinal OCTN1 and OCTN2 protein expressions

Using Western blots, we determined OCTN1 and OCTN2 protein levels in human ileal and colonic biopsies. A representative example is shown in Fig. 1. The expression of OCTN1 protein was significantly higher in the ileum (n = 31) compared to the colon (n = 16) (p < 0.0002) (Fig. 2A). This higher expression was also found in all 3 subgroups: CD (p = 0.001), UC (p = 0.009) and controls (p = 0.001). The expression of OCTN2 protein was not found to be statistically different in ileal (n = 35) compared to colonic biopsies (n = 18) (Fig. 2B).

The expression of OCTNs protein levels were then compared between CD, UC and control groups. OCTN-1 levels tended to be higher in UC patients’ ileal tissue (n = 5) compared to that from CD patients (n = 17) or controls (n = 9) (Fig. 2C). The differences, however, were not statistically significant, given the low numbers of patients in the subgroups. There was also a trend towards lower OCTN2 protein expression in ileal tissue of CD (n = 19) compared to UC patients (n = 6) or controls (n = 10). OCTN2 protein expression, in colonic biopsies only, tended to be lower in UC (n = 2) than in CD patients (n = 7) or controls (n = 11). However, these differences did not reach statistical significance, again due to low numbers of patients (Fig. 2D).

We then compared the expression of OCTN1 and OCTN2 proteins (as described above) according to the genotype of common OCTN polymorphisms (Fig. 3). The expression of OCTN1 protein in CD patients was significantly higher (p < 0.05) in the homozygous TT group (n = 8) compared to the wild type CC group (n = 5) (Fig. 3A). Similarly, the expression was significantly higher (p < 0.025) when both mutant heterozygous and homozygous genotypes were combined (n = 13) (Fig. 3C). For OCTN2, there was a trend showing higher expression in CD patients (p < 0.1) in the homozygous CC group (n = 4) compared to the wild type GG (n = 6) or heterozygous CG groups (n = 4) (Fig. 3B). This trend was also observed if mutant heterozygous and homozygous genotypes are combined (n = 8) (Fig. 3D). No significant difference was seen in controls for both OCTNs proteins. When all patients were pooled (CD and control) (n = 14) compared to the wild type CC group (n = 7) (Fig. 3E). For OCTN2 no statistical difference was observed between homozygous CC groups (n = 8) compared to the wild type GG (n = 11) (Fig. 3F).

3.4. Carnitine transport by intestinal BBM

Maximal transport was very rapidly reached (10 s) and was found to be predominantly sodium dependent (two fold higher) (Fig. 4A). Carnitine transport in CD (n = 10) and control (n = 10) patients’ tissue (Fig. 4B) was not found to be different (0.454, 0.508 nM of carnitine/mg of protein/min, respectively).

3.5. Carnitine transport as function of OCTN polymorphisms

We then evaluated intestinal BBM carnitine transport between groups of patients with or without OCTN1 or OCTN2 polymorphisms (Fig. 5). We compared the analysis of OCTN1 wild type genotype with mutant heterozygous and homozygous genotypes. In view of the relatively small number of individuals with polymorphisms of OCTN1, we combined data for heterozygous and homozygous mutations into one group. Transport studies were not available for OCTN2 mutant homozygous genotypes. Therefore, a comparison was only possible for wild type versus mutant heterozygous genotypes. Carnitine transport tended to be higher in tissue from patients with mutant heterozygous (TC) OCTN1 genotypes compared to wild type (CC) genotype, irrespective of the presence or absence of CD (Fig. 5A). When mutant...
homozygous (TT) and heterozygous (TC) genotypes were pooled, a similar tendency was observed (Fig. 5B). The same findings were observed for OCTN2 between wild type (GG) and mutant heterozygous (CG) genotypes (Fig. 5C), although the difference reached statistical significance ($p=0.001$) for the control group.

4. Discussion

As anticipated, both OCTN1 and OCTN2 proteins were detected in the mucosa of human intestine.\textsuperscript{24,27} Higher expression of OCTN1 in the ileum compared to the colon (Fig. 2A) confirms a previous report.\textsuperscript{25} Given that OCTNs transport a variety of substances including nutrients, it is not surprising that they are present in a larger amount in the small bowel. However, we did not observe a difference for OCTN2 protein expression between colonic and ileal tissues (Fig. 2B). This may reflect lower OCTN2 expression in the gut than in the kidney, where it has a predominant role in transporting cations.\textsuperscript{23}

One of the aims of this research was to compare the intestinal expression of OCTN proteins between IBD and controls. No significant differences were found between CD, UC and control groups, for either ileal or colonic biopsies (Fig. 2C, D). It is important to note that the biopsies in this study were intentionally taken from macroscopically normal appearing areas of bowel. OCTN2 protein expression tended to be lower in colonic tissue from UC compared to CD and controls (Fig. 2D). Although our observation was non-significant, two groups observed similar findings. D’Argenico et al. found that OCTN2 was less transcribed and expressed in colonic tissue in rats with trinitrobenzene sulfonic acid (TNBS) experimental colitis\textsuperscript{38} and Wojtal et al. found also modified expression of several transporters in intestinal tissue of IBD patients. They showed that OCTN2 mRNA levels were lower in ileal specimen of IBD patients compared to controls.\textsuperscript{39}

The expression of OCTN protein was then analyzed as a function of the presence of genetic polymorphisms. The frequencies of polymorphisms found (Table 2) were similar to those published.\textsuperscript{40} We found that intestinal OCTN1 expression was significantly higher in CD patients with homozygous polymorphisms, compared to controls (Fig. 3A). The effect of any point mutation depends on its localization in the gene. It can alter the function of the protein, modify its migration or insertion in the mucosa, or can have no effect. Our results overall suggest that individuals with OCTN mutations have a compensatory increase in expression of the transporter protein.
Another aim was to compare the function of OCTNs (carnitine transport) between IBD and controls and to correlate the results with the corresponding genetic polymorphisms. We did not find any difference in carnitine transport between IBD and control groups (Fig. 4). This could be explained by the fact that in our cohort, despite the fact that OCTN mutations are more frequent in CD patients, the number of mutations was found in equal frequency between CD patients and control groups in the cohorts studied. A trend to higher carnitine transport was observed when analyzed by the presence or absence of OCTN1 mutations (mutant heterozygous or combined mutant heterozygous and homozygous), irrespective of the presence or absence of CD (Fig. 5A and B). Similar findings were shown for OCTN2, reaching significance for controls (Fig. 5C). The fact that the number of patients in the different subgroups was low probably explains why much of the data failed to reach statistical significance.

Few studies have examined the functional effects of OCTN polymorphisms. Peltekova et al. reported diminished transport of carnitine by the transfected mutated OCTN1. It was proposed that OCTN mutations might impair the transporter’s migration to the apical membrane, modifying its transport capacity. In an effort to determine the physiological role of OCTN1, Kato et al. constructed a gene knockout mouse (OCTN1−/−). Metabolome analysis revealed a complete deficiency of the antioxidant ergothioneine. These knockout animals were reported by the same group to have more intestinal inflammation using an ischemia reperfusion model. Interestingly, they also found that patients with CD were found to have lower ergothioneine concentrations. These findings support a potential protective effect of OCTN function in preventing intestinal tissue injury. In contrast, Taubert et al. recently reported higher ergothioneine intestinal tissue concentrations in CD patients with 503F variant of OCTN1. Bene et al. did not find any effect of OCTN1 and OCTN2 polymorphisms on plasma carnitine-ester status in CD.

In the increasingly complex genetic background of CD, the IBD5 locus has been a focus of interest without a clearly understood functional significance. This study is the first to examine intestinal carnitine transport in human intestinal tissue, comparing CD patients to controls. The data herein reveal that OCTN protein levels appear to be similar in intestinal tissue from CD patients and controls and that, overall, ileal carnitine transport is similar in CD and control groups. Further analyses are underway in a larger cohort to confirm these findings.

Conflict of interest

All the authors declare having no conflict of interest.

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MG did the patients recruitment, the scientific work and wrote the manuscript under supervision of SD, IE and EGS. PG, JR, AB and PC actively participate to the patient recruitment. EL and IQ participate in the conception of the study. All the authors were implicated in the revision and approved the manuscript.

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