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Original Article

Differential expression of TLR2, TLR4 and JNK in mucosa of ileal pouches for ulcerative colitis. Is there a role for bacterial antigen pathway in asymptomatic patients?

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Abstract: Introduction: Ileal pouch-anal anastomosis (IPAA) is the preferred surgical procedure for patients with refractory ulcerative colitis (UC) and familial adenomatous polyposis (FAP). However, pouchitis is the most common complication after IPAA in UC patients and only occurs after ileostomy closure. Therefore, it is important to get more information about the role of the ileal pouch microbiota and mucosa susceptibility to inflammation in UC patients. Therefore, we evaluated Toll-like receptors (TLRs) expression in normal endoscopic and histological mucosa of the ileal pouch in patients with UC and FAP, in order to find any abnormality in this pathway in asymptomatic patients, which may contribute to pouchitis. Materials and Methods: Twelve patients (six with UC and six with FAP) with "J" pouch reconstruction, after total rectocolectomy, were studied. Biopsies were obtained from the mucosa of the pouch. Normal ileum biopsies were obtained from six patients submitted to ileocolonoscopy with no abnormalities. The specimens were snap-frozen and the expressions of TLR2, TLR4 and JNK (nuclear signalization factor) were determined by immunoblot protein extract. Results: Patients with UC had significantly higher protein levels of TLR4 than controls and FAP. The expressions of TLR2 and JNK were similar in the groups. Conclusion: Patients with UC had higher levels of TLR4, even in the absence of clinical, endoscopic and histological pouchitis. These findings may explain a tendency towards the up-regulation of intracellular pathways activated by bacterial antigens in UC patients, which could contribute to the production of proinflammatory mediators and pouchitis development.

Keywords: toll-like receptors, cytokines, ileal pouch, pouchitis, ulcerative colitis, familial adenomatous polyposis

Introduction

Ileal pouch-anal anastomosis (IPAA) is the elective procedure of choice in the surgical management of refractory ulcerative colitis (UC) [1-3]. Although the procedure improves the health-related quality of life and substantially reduces the risk for ulcerative colitis-associated dysplasia or cancer, complications are common. Pouchitis is a syndrome of unknown etiology and it may affect up to 50 percent of patients who undergo IPAA for UC. Pouchitis occurs with a much lower incidence in patients with familial adenomatous polyposis (FAP), suggesting that constitutive differences between UC and FAP pouches have a critical role in its pathogenesis [4, 5]. This complication only develops after ileostomy closure, when the pouch mucosa starts to be exposed to the fecal stream [6, 7]. Manipulation of microflora with antibiotic or probiotic agents in patients with pouchitis often achieves a therapeutic effect [8, 9], as also observed in active colitis of UC patients [10].

Innate mucosal immunity, which mediates bacteria–host interactions, consists of multiple components, including epithelial barrier function and the mucous gel layer, Toll-like receptors (TLRs), dendritic cells, macrophages, and Paneth cells with their antimicrobial peptides. Many studies have indicated that a complex interplay of genetic, microbial, and environ-

mental factors culminates in sustained aberrant intestinal innate immunity [1, 11].

TLRs are members of the pattern recognition receptor family (PRRs) and play a central role in the initiation of innate cellular responses and the subsequent adaptive immune response to a variety of pathogens [12]. They can be grouped into two main categories: cell surface receptors and receptors localized in the endosome. It is important to make this distinction because surface Toll-like receptor 2 (TLR2) binds molecules on the bacterial or yeast cell wall or lipopeptides from Gram-positive bacteria and Toll-like receptor 4 (TLR4) can be activated by lipopolysaccharide (LPS), an endotoxin which is produced by Gram-negative bacteria, whereas endosomal TLR, which are activated by microbial nucleic acids, are less readily accessible. This difference in subcellular localization translates into distinct functions within the antimicrobial immune response [13]. Host organism responses are activated when microbial components are recognized by a variety of pathogen sensors, particularly TLR4, initiating the intracellular signal cascade that culminates in c-Jun N-terminal kinases (JNKs) activation and translocation of transcription factors to the nucleus and the biosynthesis of inflammatory cytokines [14-16] and modulation of defensin expression [17]. Moreover, LPS-induced signaling through TLRs rapidly leads to NF-KB activation and cytokine expression in monocytes [12].

High expressions of TLR2 and TLR4 in the colon mucosa of UC patients have been identified, showing that intestinal microorganisms play a role in the initiation and maintenance of disease [18-21]. Indeed, TLR4 and TLR2 are important receptors involved in signaling pathways during the development of experimental colitis [22-24], and TLR4 gene mutations and polymorphisms have been associated with ulcerative colitis disease [25, 26]. However, TLRs signalization pathways in the ileal pouch are not well characterized and there are few studies in the literature that have evaluated this putative role of pouchitis development [27-29].

As such, in order to find any abnormality in this pathway in asymptomatic patients, TLR expressions were compared in the asymptomatic ileal pouch mucosa of highly pouchitis-prone UC patients and pouchitis-protected patients with FAP. For this purpose, we employed immunoblotting assays to determine the expressions of TLR2, TLR4 and JNK in ileal pouch biopsies.

Material and methods

Mucosal biopsies were obtained from six patients with non-inflamed IPAA after rectocolectomy for UC [median age, 50.5 (range, 36-63) years; 50% male; 50% female], and six patients with non-inflamed IPAA after rectocolectomy for FAP [median age, 35.5 (range, 21-59) years; 50% male; 50% female]. The follow-up after the operation was 87 (42-168) months. The reservoir design was of the "J" type in all patients, and the right colon vascular arcade was preserved as a supplementary blood supply to the terminal ileum [30]. Mucosectomy was performed, with hand-sewn ileo-anal anastomosis. The patients had had their ileostomy closed for more than one year, at the time of the study. The absence of pouchitis was defined clinically, histology and endoscopically, according to the PDAI [31]. The control group was composed of six individuals with normal colonoscopy examination, with a median age of 57.3 (range, 41 – 63) years and 50% were female. Six biopsies from each patient were obtained from the terminal ileum (control) and from the ileal pouch (UC and FAP).

The study was performed in accordance with the Declaration of Helsinki and was approved by the local ethical committee. All biopsies were taken after informed consent from the patients. The study was carried out at the State University of Campinas, Coloproctology Unit, and at the Cell Signaling Laboratory of the Department of Internal Medicine.

Mucosal biopsies from the pouches and from normal ileum were snap-frozen in liquid nitrogen and stored at -80^{o}C until use. For total protein extract preparation, the fragments were homogenized in solubilization buffer at 4^{o}C [1% Triton X-100, 100mM Tris-HCl (pH 7.4), 100mM sodium pyrophosphate, 100mM sodium fluoride, 10 mM EDTA, 10mM sodium orthovanadate, 2.0mM phenylmethylsulfonyl fluoride (PMSF), and 0.1 mg aprotinin/ml] with a Polytron PTA 20S generator (model PT 10/35; Brinkmann Instruments, Westbury, NY) operated at maximum speed for 30 sec. Insoluble material was removed by centrifugation (20 min at 11,0000 rpm at 4^{o}C). The protein concentrations of the supernatants were determined by the Bradford dye binding method [32]. Aliquots

of the resulting supernatants containing 100 µg total proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes and blotted with anti-TLR2, anti-TLR4 and anti-pJNK antibodies [33].

Reagents for SDS-PAGE and immunoblotting were from Bio-Rad Laboratories (Richmond, CA). Phenylmethylsulfonyl fluoride, aprotinin, Triton X -100, Tween 20, glycerol were from Sigma (St. Louis, MO). Nitrocellulose paper (BA85, 0.2µm) was from Amersham (Aylesbury, UK). The anti-TLR2 (sc-16237, rabbit polyclonal) and anti-TLR4 (sc-10741, rabbit polyclonal) antibodies were purchased from Santa Cruz Biotechnology®, Inc. (Santa Cruz, CA). The anti-phospho-SAPK/JNK (sc-5559, rabbit polyclonal) was purchased from Cell Signaling Technology®, Inc. The signal was detected by a chemiluminescent reaction (SuperSignal®West Pico Chemiluminescent Substrate from Pierce Biothecnology, Inc. Rockford).

All numerical results are expressed as the mean ± SEM of the indicated number of experiments. The results of blots are presented as direct comparisons of bands in autoradiographs and quantified by densitometry using the Gel-Pro Analyzer 3.1 software (Exon-Intron Inc., Farrell, MD). Data were analyzed by repeated-measure ANOVA (one-way or two-way ANOVA) followed by analysis of significance (Tukey-Kramer Multiple Comparisons test), comparing UC, FAP, and control groups. The level of significance was set at $p<0.05$.

Results

The level of TRL4 expression was statistically higher in operated patients with UC when compared with the FAP and Control groups ($p<0.05$). The expression of TLR2 was similar among the groups ($p<0.05$); however there was a tendency towards higher levels in UC patients, when compared to the other groups ($p=0.12$). Similarly, local levels of JNK were similar in the pouches of the UC, FAP patients and Control ($p>0.05$).

Protein expression determinations are shown in **Figures 1, 2** and **3**.

Discussion

The etiology of primary pouchitis remains unclear, and this has precluded the development of appropriate prophylaxis and treatment. A previous diagnosis of UC seems to be important in this disease, as demonstrated by the higher frequency of this postoperative complication in UC patients. The fecal stream and stasis are involved in the pathogenesis of immunological reactions in the ileal pouch, but do not explain the difference in the incidence of pouchitis in UC and FAP patients. Immunological changes occur in the pouch for at least one year after ileostomy closure, and are considered to be adaptive changes [1, 11, 34].

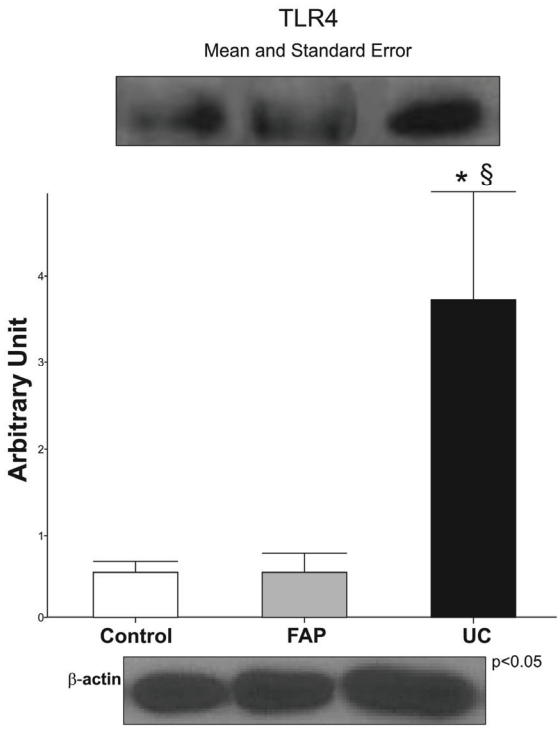

Figure 1 Representative Western blot analyses and determination of TLR4 protein expression in non-inflamed pouches in the Control, FAP and UC groups. For illustration purpose each line band represents one patient. For all conditions, n=06, *$p<0.05$ vs Control; § $p<0.05$ vs FAP.

Efficient immune responses depend upon a close interaction between the innate and adaptive immune systems [14]. The role of the commensal intestinal microflora in colitis has been studied in a number of experimental models, but detailed knowledge regarding the gut microbiota composition in acute intestinal inflammation is still limited [34]. Recently, Heimesaat et

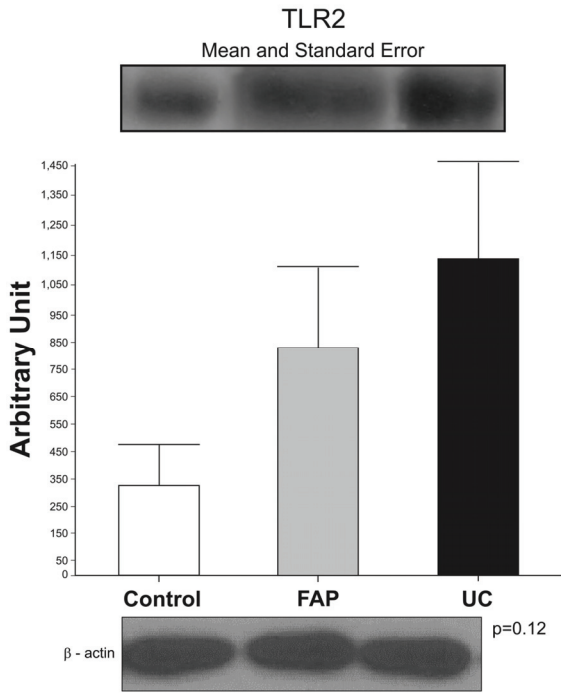

Figure 2 Representative Western blot analyses and determination of TLR2 protein expression in non-inflamed pouches in the Control, FAP and UC groups. For illustration purpose each line band represents one patient. For all conditions, n=06, *p<0.05 vs Control; § p<0.05 vs FAP.

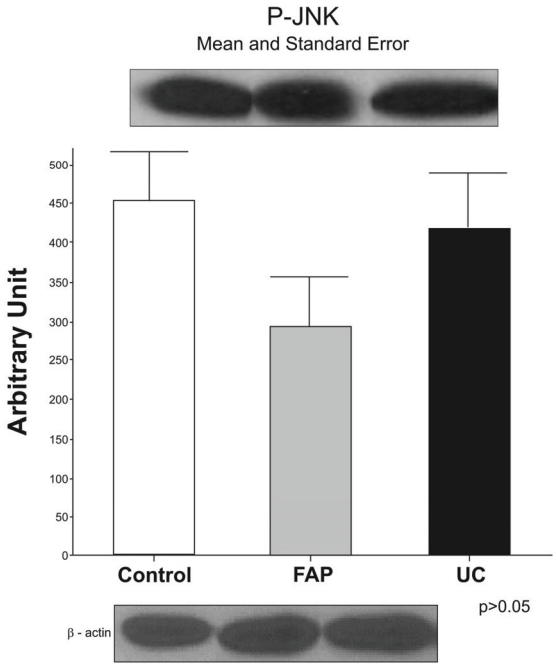

Figure 3. Representative Western blot analyses and determination of JNK protein expression in non-inflamed pouches in the Control, FAP and UC groups. For illustration purpose each line band represents one patient. For all conditions, n=06, *p<0.05 vs Control; § p<0.05 vs FAP.

al [34] demonstrated that acute murine ileitis is accompanied by a rigorous *E. coli* overgrowth in the terminal ileum and Liu et al [35] showed that TLR4 monoclonal antibody blockade suppresses colitis under experimental conditions. Bambury et al [11] identified significant differences in bacterial colonization between UC and FAP pouches. Shortly after stoma reversal and pouch function, a qualitative switch occurs in bacterial colonization. Whereas facultative anaerobic species predominate in the end-ileostomy of patients with UC, strict anaerobe species predominate in the UC pouch. Sulphate-reducing bacteria (SRB) are found with increasing frequency in the stools of patients with active UC, and colonize pouches fashioned for UC, but not those fashioned for FAP. These findings indicate that pouchitis may be linked to SRB colonization of the ileal pouch [1, 11].

Pro-inflammatory cytokines have been reported in ileal pouches; TNF-α, IL-1β, IL-6, IL-8, IFN-γ expressions are elevated in UC patients pouchitis [36-39]. Furthermore, it has been suggested that a high expression of cell signaling factors, such as STAT-1, in the ileal pouch mucosa may be similar to those found in active UC [3, 40]. The TLRs pathway is an important inflammatory mechanism in the pathogenesis of inflammatory bowel diseases, and TLRs are considered a biomarker of chronic inflammation [41]. TLRs are necessary for maintaining tolerance and eliminating pathogenic microorganisms under healthy conditions; however these proteins can amplify inappropriate immune responses, which cause chronic inflammation [42]. Recent cell culture experiments, using macrophages stimulated with bacteria and TLR ligands, revealed a specific defect in the TLR4 response in UC, when compared to controls, demonstrating the over-expression of molecules associated with leukocyte recruitment and activation [43]. The TLR5 protein recognizes various molecules of the microbiota, including the principal protein of pathogenic bacteria (flagellin). A important study showed decreased TLR5 expression in the mucosa of UC patients [44], indicating that LPS

bacterial antigen could be the main bacterial product involved in inflammatory aspects and host-bacteria interations in UC [45]. However, few studies have evaluated the immunological activity in ileal pouches, particularly the interactions between bacterial antigens and the intestinal mucosa, and whether there is a tendency for inflammation in asymptomatic patients with ileal pouches.

Toyama et al [28] showed that TLR2 expression is upregulated in pouchitis and TLR4 expression is increased both in the normal pouch and in pouchitis, as compared with the normal ileum, but these expressions were not compared with FAP patients and a total extract of proteins was not available. A study performed using semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) discovered alterations in mRNA levels of TLRs (TLR3 and TLR5) in pouchitis. Indeed, TLR3 expression was decreased, while TLR5 expression presented high levels in the normal pouch mucosa of UC, compared with normal ileal mucosa [29]. A combined carriership of the TLR9-1237C and CD14-260T allele was found to be linked to the development of chronic pouchitis [27].

Ileal pouch and pouchitis have been considered a model to study inflammatory bowel disease, because they offer the opportunity to evaluate bacterial background and host-bacterial interactions in a sequence, even in the absence of clinical and endoscopic inflammation [1, 9].

In the present study, we evaluated the expressions of TLRs and JNK proteins to address intracellular pathways activated by bacterial antigens in normal ileal pouches. Even in such optimal clinical, endoscopic and histological conditions, the local levels of TLR4, were high in UC patients, and TLR2 demonstrated a discrete tendency to have higher expression in UC patients, when compared to FAP. This finding indicates that there is an up-regulation of the bacterial receptor on the cell membrane surface, especially on those cells which respond to lipopolysaccharide (LPS) in UC. This receptor expression could lead to or correlate with the tendency towards inflammation in these pouches. It is probable that the bacterial population is responsible for initiating and propagating inflammatory conditions, although, this is probably due to the higher expression of proteins on the surface of the cell, rather than due to increases in bacteria. TLR4, TLR2 and JNK expressions were found to be similar in FAP patients, when compared to control individuals, demonstrating that FAP patients do not have demonstrate a tendency towards pouch inflammation, when considering inflammation related to bacterial products.

Based on cell culture studies, JNK is reported as an important regulator of the release of cytokines by immunocompetent cells in inflammatory bowel diseases and is activated by LPS and other bacterial products, cytokines such tumor necrosis factor (TNF-alfa) and interleukin (IL-1), and growth factors [46]. Blockade of the JNK pathway with JNK inhibitors in animal models of inflammatory bowel disease led to the resolution of intestinal inflammation [47], however there are, currently, no data regarding JNK in the ileal pouch. With regard to the similar JNK expression in the different groups of our study, its findings could indicate that all patients were asymptomatic with normal endoscopic and histological features; as such, there is still a balance between pro and anti-inflammatory pathways and a macroscopic inflammation has not installed.

An understanding of the role of bacteria in the ileal pouch may provide more information about the molecular biology involved in the normal ileal pouch and pouchitis, and in the primary diseases, FAP and UC, in association with the different outcomes of these conditions.

In summary, the present study shows that, even in the absence of clinical, endoscopic and histological pouchitis, patients with UC had higher levels of TLR4 membrane receptor, when compared to FAP and control groups. These findings may suggest a tendency towards an up-regulation of the intracellular pathways activated by bacterial products in UC patients, which could contribute to the release of proinflammatory cytokines and, ultimately, lead to pouchitis.

Competing interests

The authors have nothing to disclose any financial or non-financial competing interests.

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