Original article

Title: *Intestinal Alkaline Phosphatase in the colonic mucosa of children with inflammatory bowel disease*

Running title: *intestinal alkaline phosphatase in pediatric IBD*

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Supportive foundations:
This work was supported by grants OTKA-76316, OTKA-K81117, and ETT-028-02. Gábor Veres and Ádam Vannay are holders of the János Bolyai Research grant; this article was supported by the János Bolyai Research Scholarship of the Hungarian Academy of Sciences.

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ABSTRACT

AIM: To investigate intestinal alkaline phosphatase (iAP) in the intestinal mucosa of children with inflammatory bowel disease (IBD).

MATERIALS AND METHODS: Colonic biopsy samples were taken from 10 healthy controls and from 15 newly diagnosed IBD patients. In IBD patients, specimens were obtained both from inflamed and non-inflamed areas. The iAP mRNA and protein expression was determined by RT-PCR and Western blot analysis, respectively. Tissue localization of iAP and TLR4 was investigated by immunofluorescent staining.

RESULTS: The iAP protein level in the inflamed mucosa of children with CD and UC was significantly decreased by 22% and 20% when compared to controls (p < 0.05, p< 0.05 respectively). Similarly, we found significantly decreased level of iAP protein in the inflamed mucosa in CD in comparison to non-inflamed mucosa in CD (p < 0.05). In addition, iAP protein level in inflamed colonic mucosa in patients with UC was decreased by 24% when compared with non-inflamed mucosa in patients with CD (p < 0.05). iAP protein levels in the non-inflamed mucosa of patients with CD were similar to controls. All patients with UC had pancolitis thus we were unable to provide non-inflamed mucosa for comparative study. iAP mRNA expression in inflamed colonic mucosa of children with CD and UC was not significantly differed in comparison to non-inflamed colonic mucosa with CD. Expressions of iAP mRNA in patients with non-inflamed mucosa and in controls were similar. Immunofluorescent staining has shown that the distribution of iAP was restricted to the epithelial surface of the colonic and terminal ileal mucosa in all groups studied. No fluorescent signal was detected in the cells of Lieberkühn crypts, in the goblet cells, and in the lamina propria’s immune cells. The colocalization of iAP with TLR4 was intensely stained with dotted-like pattern. The iAP was present in the inflamed and non-inflamed mucosa of patients with CD, UC, and in control biopsy specimens irrespectively, whether it was stained in the terminal ileum or in the colon. However, the fluorescent signal of TLR4 was more pronounced in the colon when compared to terminal ileum in all groups studied.

CONCLUSION: Lower than normal iAP protein levels in inflamed mucosa of IBD patients may indicate the role of iAP in inflammatory lesions of IBD. Based on our results, administration of exogenous iAP enzyme to patients with active form of IBD may be supplemental therapeutic option.
KEY WORDS: intestinal alkaline phosphatase, Toll-like receptor, colonic biopsy, children, inflammatory bowel disease
INTRODUCTION

The etiology of inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn’s disease (CD), still remains unclear. It is hypothesized that in genetically susceptible individuals the inappropriate and ongoing activation of the mucosal immunity against luminal antigens is a major player responsible for the inflammation [1, 2]. In active IBD the tolerance towards the resident intestinal flora is decreased. The balance between protective and commensal luminal bacterial species is lost and due to increased mucosal permeability and insufficient mucosal clearance, the commensal flora and pathogenic bacteria enter into the lamina propria and the destructive inflammatory responses are unavoidable [3-6]. This prompts an exaggerated immune response with the activation of the two arms of mucosal immune system, the innate and adaptive elements [7, 8].

The activation of innate immune system heavily depends on the recognition of microbes by pattern recognition receptors such as Toll-like receptors (TLRs). The TLR family consists of 13 members, and each has different type of ligands. One of them is TLR4, which is responsible for recognition of lipopolysaccharide (LPS), a principal component of bacterial outer membrane. Uncontrolled activation of TLR4 may lead to the loss of mucosal barrier integrity, aggravation of inflammatory response within the gut epithelial mucosa, increased expression of TLR-ligands and tumorgenesis [9-13]. Previously we found increased TLR4 protein and mRNA levels in the inflamed mucosa of children with IBD and celiac disease [14, 15].

An increasing body of evidence also supports the regulatory role of intestinal alkaline phosphatase (iAP) in TLR activation. iAP is expressed on the apical surface of enterocytes and exists in membrane-bound and soluble forms [16]. iAP plays an essential role in the inactivation of LPS through dephosphorylation its lipid A moiety, thus generating a non-toxic monophosphoryl section. This dephosphorylated monophosphoryl lipid A is not able to form a complex with TLR4 [17, 18].

There is only one human study where expression of iAP (mRNA) in adult IBD patients was analyzed and lower than normal iAP mRNA expression was found in patients epithelial specimens [19]. It should be noted, however, that no data on the level of iAP protein in IBD mucosa is available. The aim of our study was to investigate iAP protein and mRNA levels in affected and non-affected colon mucosa of children with newly
diagnosed IBD. In addition, our secondary aim was to determine the localization of iAP enzyme with TLR4.
MATERIALS AND METHODS

Patients and colonic biopsies
Ten children (7 boys, 3 girls; median age: 10.5 years, range: 1.5-15 years) with newly diagnosed CD and 5 children (3 boys, 2 girls; median age: 11 years, range: 6-17 years) with newly diagnosed UC and 10 control children (5 boys, 5 girls; median age: 9.5 years, range: 1.5-16 years) were enrolled in the study (for clinical data, see Table 1). IBD was diagnosed according to Porto criteria [20, 21]. The presenting symptoms in CD were perianal fistula, hematochezia, abdominal pain, diarrhea-bloody diarrhea, or anemia. All of patients later diagnosed with UC had hematochezia, some of them had abdominal pain and weight loss. Colonic biopsy samples were taken from macroscopically inflamed and non-inflamed sites of the colonic mucosa in children with CD. As each UC children has pancolitis, just inflamed mucosa was obtained in UC (Table 1). The activity score was calculated by means of PCDAI (Pediatric Crohn’s Disease Activity Index) and PUCAI (Pediatric Ulcerative Colitis Activity Index) [22, 23]. Measuring disease activity in pediatric CD is based on disease history (abdominal pain, stools per day and general well-being), laboratory findings, weight, abdominal- and perianal examination, extra-intestinal manifestations, and height velocity [24]. PUCAI requires no laboratory values [25]. The mean PCDAI of our patients was 33.75, the mean PUCAI was 35. This means that both groups belonged to moderate to severe disease activity. Control children were referred to the outpatient clinic due to rectal bleeding, constipation or weight loss. Colonoscopy was the part of their diagnostic procedure and the biopsy specimens showed normal macroscopic appearance and histology. Written informed consent was obtained from parents prior to the procedure, and the study was approved by the Semmelweis University Regional and Institutional Committee and Research Ethics.

RNA isolation and real-time PCR
Total RNA was isolated from the colonic biopsy samples by RNeasy Total RNA Isolation Kit (Qiagen GmbH, Hilden, Germany), according to the instructions of the manufacturer. One µg of total RNA was reverse-transcribed and iAP mRNA expressions were determined by real-time PCR on Light Cycler480 (Roche Diagnostics, Mannheim, Germany). PCRs were performed containing RealTime ready Catalog Assay primer (Roche Diagnostics), Lyght Cycler 480 Probes Master (Roche Diagnostics, Mannheim, Germany), and cDNA. Conditions for iAP mRNA measurements: one cycle, 95°C, 10 min
(denaturation), followed by several cycles at 95°C, 10 s and 30 sec, 72°C 1 sec (annealing and extension). The mRNA expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as internal control was determined using Brilliant II Fast SYBR Green QPCR Master Mix (Stratagene, Cedar Creek, TX, USA). PCR primers (Forward: 5-CAC CAC CAT GGA GAA GGC TG-3'; Reverse: 5-GTG ATG GCA TGG ACT GTG-3', Invitrogen, CA, USA) and cDNA. Conditions for GAPDH: one cycle, 95°C, 2 min, 50 cycles at 95°C 20 sec and 60°C, 40 sec. Results were analyzed by Light-Cycler software 480 (Roche Diagnostics).

**Protein isolation and Western blotting**

Colonic biopsy specimens were homogenized in lysing solution and protein concentrations were determined by DC Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). Half μg protein from each sample was separated by 10% sodium dodecyl sulfate-polyacrilamide gel electrophoresis (120V, 40mA, 120 min) (PenguinTM Dual-Gel Water Cooler Systems, Owl, NH, USA) and transferred to nitrocellulose membrane (GE Healthcare, Little Chalfont, UK) (70V, 220mA, 120 min) (MiniTankTM electroblotter, Owl). Membranes were blocked in 1% non-fat dry milk solution (1h) and incubated with iAP specific rabbit polyclonal antibody (1:1000, 1h) (AbCam, Cambridge, UK). Equal protein loading was confirmed by β-actin specific (C-11) goat polyclonal IgG antibody (1:100) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Peroxidase-conjugated secondary anti-rabbit IgG or donkey anti-goat IgG antibodies (1:2000, 30 min) (Santa Cruz Biotechnology Inc.) were used. Immunoreactive bands were visualized using the enhanced chemiluminescence Western blotting detection protocol (GE Healthcare). Bands were analyzed with software Image J. 1.42q (National Institutes of Health, USA). The values were expressed as relative optical density.

**Immunofluorescent staining**

Biopsy samples were snap-frozen, embedded in Shandon cryomatrix (ThermoElectron Co., Waltham, USA), cut to 3-4 μm slides and double incubated with TLR4 specific goat polyclonal antibody and iAP specific rabbit polyclonal antibody (1:100, 1 h) (Abcam Plc). Secondary antibodies were Alexa Fluor 488 donkey anti-goat and Alexa Fluor 568 goat anti-rabbit antibodies (Invitrogen). Zeiss LSM 510 Meta confocal laser scanning microscope (Carl Zeiss, Jena, Germany) was used with 20x Plan Apochromat (NA=0.8) and 63x Plan Apochromat oil immersion differential interference contrast objectives
(NA=1.4).

**Statistical analysis**

Data were analyzed using Statistica 7.0 software (StatSoft Inc., Tulsa, OK, USA). After testing the normality with Shapiro-Wilk’s test, non-parametric Mann-Whitney U-test was used. Data were considered statistically significant if p≤0.05, and expressed as median interquartile range (IQR).
RESULTS

iAP protein levels
Western blot analysis revealed one distinct band at 60 kDa. iAP protein level in the inflamed mucosa of children with CD and UC that was lower by 22% and 20%, respectively, compared to controls (p<0.05). We found lower iAP protein level in the inflamed mucosa in CD compared to non-inflamed mucosa in CD (p<0.05). iAP protein level in inflamed colonic mucosa in UC patients was decreased by 24% compared with non-inflamed mucosa in CD patients (p<0.05) (Figure 1). iAP protein levels in the non-inflamed mucosa of patients with CD were normal.

iAP mRNA expression
iAP mRNA expression in inflamed and non-inflamed colonic mucosa in IBD was comparable to that in controls (Figure 2).

Mucosal localization of iAP and TLR4
Distribution of iAP was restricted to the epithelial surface of the colonic and terminal ileal mucosa in each group. No fluorescent signal was detected in Lieberkühn crypt cells, in goblet cells, and in lamina propria’s immune cells. The co-localization of iAP with TLR4 was intensely stained with dotted-like pattern. iAP was present in inflamed and non-inflamed mucosa of patients with CD, UC, and in control specimens irrespectively, whether it was stained in the terminal ileum or in the colon. However, the fluorescent signal of TLR4 was more pronounced in the colon when compared to terminal ileum in all groups (Figure 3).
DISCUSSION

A dysregulated immune response, involving the innate immunity in the intestinal mucosa plays a role in the pathomechanism of IBD. The maintenance of microbiota and host is relieved by the balance of microbiota and immune activation that may be disturbed in IBD [26]. Previously we and others showed that activation of TLR4 by bacterial LPS contributes to disease progression [14, 27].

Recently, in connection of LPS activated TLR4, a new enzyme, iAP has received an increasing attention as a factor responsible for mucosal defense. iAP dephosphorylates and detoxifies LPS and, hence, generates an inactive, non-toxic form. This may be one of key factors why dephosphorylated LPS is unable to bind to TLR4 and the innate immune system is not triggered. iAP may control the interaction between TLR4 on intestinal mucosa and LPS derived from the bacterial flora [28, 29].

In the present study we obtained data regarding the protein level, mRNA expression and localization of iAP in the intestinal mucosa of children with IBD. Lower than normal iAP levels was observed in the inflamed mucosa of CD and UC patients. Previously it was hypothesized that the altered LPS-dephosphorylating activity may be consequence of decreased iAP activity. We think that in accordance of Tuin et al. our observations also suggest that iAP has role in the pathogenesis of IBD. Decreased iAP levels of the inflamed mucosa may be associated with decreased LPS detoxication and, consequentially, with increased TLR4 activation. On the other hand we found no significant difference in iAP mRNA expression that may indicate the possible role of posttranscriptional regulation.

According to the literature Tuin et al. demonstrated decreased iAP mRNA expression of pretreated CD patients compared to controls [19] However, it should be noted that in this study more than half of the patients received immunosuppressive drugs such as infliximab, methotrexate, corticosteroids, and thiopurine at the time of sample collection, which may influence the iAP mRNA synthesis [30]. The unique feature of our study is the investigation of children without prior immune modulatory therapy, hence, our results can be considered as characteristic for IBD.

Previously, we have demonstrated increased TLR4 mRNA expression and protein levels in the inflamed colonic mucosa of children with IBD [14] Therefore, the finding that iAP
and TLR4 are co-localized, is particularly important from two aspects. First, it supports a linked role of iAP in the maintenance of mucosal integrity both in healthy and in diseased subjects. Second, the lower than normal iAP in the presence of a higher than normal TLR4 expression might indicate a disbalance in iAP / TLR4 that would result in an increased susceptibility of mucosa to LPS. Indeed, this mechanism is already demonstrated in animal models of induced colitis. Our results are the first that indicate the presence of this phenomenon in the man.

The current management of IBD consists of conventional therapy, but in severe therapy resistant cases requires more powerful therapies, such as biological treatment [31]. The therapeutic manipulation to restore the balance of microflora may have a strong impact on mucosal healing of IBD [32]. In animal models of dextrane sodium sulfate induced colitis (DSS-colitis) exogenously administered iAP improved the macroscopical and microscopical signs of the colitis [33]. Microscopic scores of DSS-induced colitis in iAP-KO were much higher than in wild-type group, which may reveal the mucosal defense role of iAP [34]. In a human study performed in adult subjects a 7-day course of iAP products decreased the activity index of therapy-resistant UC patients [35]. Oral administration of iAP may have a beneficial effect in case of severe intestinal epithelial damage [36]. Based on our results obtained in pediatric population might indicate a similar approach in children with IBD.

In summary, to the best of our knowledge, we have firstly demonstrated the decrement of iAP enzyme in the mucosa of patients with IBD. Decreased level of iAP with reduced LPS-detoxifying capacity could be responsible for increased bacterial passage across the intestinal mucosa of patients with IBD and this may play an important role in the pathogenesis. In addition, co-localization of iAP and TLR4 was demonstrated in the epithelial compartment. Based on our results, administration of exogenous iAP enzyme to patients with active form of IBD may be supplemental therapeutic option.
ACKNOWLEDGEMENT, CONFLICT OF INTEREST
We are grateful for the excellent technical assistance of Mária Bernáth. The authors declare that they have no conflict of interest.

COMMENTS

Background
This is a well-written manuscript reporting about significance of intestinal alkaline phosphatase in the colonic mucosa for the pathogenesis of inflammatory bowel disease in children.

Research frontiers
The manuscript contains clear component of novelty - to the best of the knowledge, the authors have firstly demonstrated the decrement of iAP enzyme in the mucosa of patients with IBD.

Innovations and breakthroughs
Decreased level of iAP with reduced LPS-detoxifying capacity could be responsible for increased bacterial passage across the intestinal mucosa of patients with IBD and this may play an important role in the pathogenesis. In addition, co-localization of iAP and TLR4 was demonstrated in the epithelial compartment.

Applications
Based on their results, authors proposed administration of exogenous iAP enzyme to patients with active form of IBD as supplemental therapeutic option. However, this hypothesis should be tested in future clinical trials.
Due to novelty of data of the manuscript I recommended publication of paper in WJG.

Terminology
The importance of mucosal barrier damage is emphasized in inflammatory bowel disease due it potential contributing factor to the pathogenesis. iAP, a potent factor to maintain or
restore mucosal barrier integrity in the gut could participate in the mucosal healing in IBD.
REFERENCES


Figures

**Figure 1. Protein levels of iAP in the colonic mucosa of children with newly diagnosed CD, UC and controls.**

Western blot analysis of the colonic biopsy specimens using iAP specific antibody reveals one distinct band at 60 kDa (A). Data for protein levels of iAP were obtained by computerized analysis of the Western blots and expressed as median IQR. (B). Analysis of significance was performed by Mann-Whitney U test. \(^{a}p<0.01\text{ vs. control}, \(^{b}p<0.05\text{ vs. non-inflamed CD}, \(^{c}p<0.05\text{ vs. control} \text{ and } \(^{d}p<0.05\text{ vs. non-inflamed CD.} \)

**Figure 2. mRNA expression of iAP in the colonic mucosa of children with newly diagnosed CD, UC and controls.**

iAP mRNA expression data were obtained by computerized analysis of PCR products. Optical density was corrected for that of GAPDH. Data are expressed as median IQR. Analysis of significance was performed by Mann-Whitney U test.

**Figure 3. Localization of iAP and TLR4 in colon of CD.**

Immunofluorescent stainig for iAP (red) and TLR4 (green) staining were performed in inflamed colon of patient with CD. Yellow colour (merge) indicates colocalization of iAP and TLR4. For the observation of non-labeled tissues differential interference contrast (DIC) was used. Nuclei are stained with blue. Bar=20μm.