

The role of intestinal alkaline phosphatase in pediatric inflammatory bowel and celiac disease

Ph.D. Thesis

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INTRODUCTION

The intestinal mucosa is not only a physical barrier against pathogens, but also a sensitive structure, which reacts strongly to environmental changes. The inappropriate immune response and mucosal barrier damage may contribute to the development of inflammatory bowel disease (IBD) and celiac disease (CD) in genetically susceptible individuals. The genetic background, pathomechanism, localization of IBD and CD is completely different, but in both diseases the development of disruption in the mucosal homeostasis and subsequent mucosal injury is essential. Alkaline phosphatase (ALP) is one of the most studied enzymes in the routine laboratory tests that occur in the intestine, liver, bone, kidney, placenta and testis. The intestinal alkaline phosphatase (iAP) regulates the absorption of fat, hydrolysis of organic phosphates, and maintains mucosal integrity of the gut.

The activation of innate immune system also depends in part on the properties of pattern recognition receptors (PRR). The Toll-like receptors (TLR) belong to the family of PRRs, which are responsible for the regeneration of intestinal epithelial barrier. The expression of TLR4 is spatially regulated, under physiological conditions it is not present in the apical surface of intestinal epithelium, so it does not create complex with LPS. iAP is particularly important in maintaining intestinal barrier integrity and triggering anti-inflammatory responses, because it is able to detoxify LPS, the component of Gram-negative bacteria membrane, and therefore the TLR-dependent inflammatory responses are not activated.

Previously our group demonstrated that TLR4 expression is increased in the intestinal mucosa of children with newly diagnosed IBD and CD compared to controls. In the colonic mucosa of adult IBD patients the LPS dephosphorylating activity was decreased, which can be explained by a reduction in iAP activity, but the amount of iAP proteins has not been studied. The positive effect of iAP on intestinal inflammation has been shown in animal models. In iAP KO mice the chemically induced colitis was histologically more severe compared to wild-type mice and iAP tablets reduced the symptoms of colitis in both investigated groups. The iAP enzyme activity was decreased in moderate and severe mucosal intestinal lesions of newly diagnosed children with CD, but in children maintained on gluten-free diet the iAP activity reached the physiological level.

According to the literature the role of iAP in pediatric IBD and CD has not yet been investigated.

AIMS

My aim was to examine iAP expression in the colonic mucosa of children with IBD and in the duodenal mucosa of children with CD. The appearance of iAP protein levels has not been studied before, although this is essential in the enzyme analysis.

During my Ph.D. work I investigated the following topics:

I. The expression of iAP and localization of iAP-TLR4 in the colonic mucosa of children with inflammatory bowel disease

1. Is there any change in the expression of iAP mRNA expression and protein level in the inflamed colonic mucosa of children with Crohn's disease (MC) and ulcerative colitis (UC), compared to controls?
2. Is there any difference in the expression of iAP mRNA and protein level in the inflamed and non-inflamed colonic mucosa of MC patients?
3. Is there any difference in the localization of iAP and TLR4 in children diagnosed with MC or UC, compared to controls?

II. The expression of iAP and localization of iAP-TLR4 in the duodenal mucosa of children with celiac disease

1. Is there any change in the expression of iAP mRNA expression and protein level in the duodenal mucosa of children with newly diagnosed CD compared to controls?
2. Is there any change in the expression of iAP mRNA expression and protein level in the duodenal mucosa of children with newly diagnosed CD and children with CD maintained on gluten-free diet (GFD) ?
3. Is there any difference in the localization of iAP and TLR4 in children with newly diagnosed CD or children with CD maintained on GFD, and controls?

PATIENTS AND METHODS

I. Patients

I.1. Group of patients with IBD and controls

Ten children (7 boys, 3 girls; median age: 10.5 years, range: 1.5-15 years) with newly diagnosed MC 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. IBD was diagnosed according to Porto criteria. The biopsy samples during colonoscopy were obtained from macroscopically inflamed and non-inflamed mucosa. Ten control children were referred to colonoscopy 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.

I. 2. Group of patients with CD and controls

Ten children (2 boys, 8 girls; median age: 4 years, range: 2-12 years) with newly diagnosed CD and 5 children (2 boys, 3 girls; median age: 12 years, range: 6-13 years) with GFD-treated CD and 10 controls (5 boys, 5 girls; median age: 9.5 years, range: 2-16 years) were enrolled in the study. CD was diagnosed by the criteria of European Society for Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN). Children on GFD had full clinical and histological remission supported by normal blood levels of tissue-transglutaminases (TG). Time frame between the first and the second biopsies of patients on GFD was 1.5 years (range 0.5–2.5 years). The control group included children with chronic abdominal pain or chronic diarrhoea referred to the gastroenterology unit of 1st Department of Pediatrics, Semmelweis University. Endoscopic examination was performed to exclude organic cause. In all cases normal blood levels of TGs were demonstrated and no histological alterations were present in their duodenal biopsy specimens.

Written informed consent was obtained from parents prior to the procedure. The study was approved by the Semmelweis University Regional and Institutional Committee and Research Ethics.

II. Real time reverse transcriptional polymerase chain reactions (RT-PCR)

One part of the duodenal and colonic mucosa biopsy specimens of the children was stored at -80°C. RNA was isolated using RNeasy™ Mini Kit according to the instructions of the manufacturer. Quality and quantity of the RNA was determined by photometry. 1 µg RNA

was reverse transcribed to cDNA. The mRNA expression changes of iAP was measured with SYBR Green based real time PCR. Gliceraldehyd-3-phosphate dehydrogenase was used as a housekeeping gene.

III. Western blot

Samples were homogenized in lysing solution, and total protein content was determined with spectrophotometry using Dc Protein Assay reagent. An equal amount of protein samples in 10% PreCast gel electrophoresis was loaded. The proteins, separated by molecular size, were transferred into nitrocellulose membranes. The membranes were blocked in 5% nonfat milk solution, then incubated with iAP specific antibody and horse radish peroxidase (HRP) conjugated secondary antibody. β -actin was used as a reference. Chemiluminescence was detected with ECLplus reagent using VersaDoc 5000MP camera. Immunoreactive bands were determined using Quantity One software.

IV. Immunofluorescent staining of iAP and TLR4

The frozen samples from duodenum, terminal ileum and colon were embedded into criomatrix, then 3-4 μ m sections were stained with iAP and TLR4 specific antibodies, then Alexa Fluor® 488 and Alexa Fluor® 568 conjugated secondary antibodies. DNA was stained with Hoechst 33342. Sections were covered with Vectashield mounting medium, and then examined with laser scanning confocal microscope.

V. Statistical analysis

The results of RT-PCR and Western blot were analyzed using non-parametric Mann-Whitney U-test. Values of $p \leq 0.05$ were considered to be significant and expressed as mean \pm SD.

RESULTS AND DISCUSSION

The change in the composition of intestinal flora and the localization of intestinal epithelium are essential in the development of mucosal inflammation and tissue destruction. The elements of innate immunity recognize the presence of bacteria, promote the formation of mucosal barrier and anti-bacterial defense response. The inappropriate immune response and the injury of mucosal barrier contribute to the development of IBD and CD. The pathomechanism and localization are completely different, but in both diseases the disruption of mucosal homeostasis and mucosal injury are essential.

Previously our group demonstrated that the TLR4 expression is increased in the inflamed colonic mucosa of newly diagnosed children with IBD and in the duodenal mucosa of patients with newly diagnosed CD, compared to controls.

In connection of LPS-activated TLR4 a new enzyme, intestinal alkaline phosphatase was paid attention, which is an important factor in the mucosal protection. Indeed, the iAP dephosphorylates the LPS, making it an inactive, non-toxic form. This dephosphorylation makes the interaction between LPS and TLR4 impossible and thus the activation of innate immune responses.

In this study the protein levels and mRNA expression of iAP, the localization of iAP and TLR4 were examined in the colonic mucosa of children with IBD and duodenal mucosa of CD patients.

I. The alteration of iAP expression in the colonic mucosa of children with IBD

So far only one human study examined the role of iAP in adult IBD patients. In this study, although the amount of iAP proteins was not measured, decreased LPS activity was found in the colon, explained by the loss of iAP activity. I first examined the iAP enzyme in therapy-naive children with IBD.

The decrease of iAP mRNA expression showed no significant difference in the inflamed mucosa of children with MC and UC, compared to non-inflamed colonic mucosa of MC patients and controls. The literature of iAP mRNA expression is controversial: the increased iAP mRNA expression in the inflamed regions of UC and MC compared to control samples, may indicate a higher production of iAP in mucosal cells. My research results are partially overlapped with the investigation of López-Posadas et al., who have shown that reactive oxygen species produced by inflamed tissues causes increased activity of iAP in

intestinal epithelial cells.

However Tuin et al. have shown that the iAP mRNA expression was reduced in the colonic mucosa of MC patients compared to controls. Although it is noted that in this study, more than half of the patients received immunosuppressive agents (infliximab, methotrexate, corticosteroids and thiopurin) at the time of sample collection, which may greatly influence the iAP mRNA synthesis. In healthy adults, higher iAP mRNA expression was found in the ileum in comparison to colon, but the iAP mRNA expression and LPS-dephosphorylation activity were significantly decreased in the inflamed mucosa of adult patients with IBD compared to non-inflamed IBD mucosa and control samples. In this study, the amount of iAP protein was not determined, and untreated and treated groups created one group of patients.

In spite of the rising tendency of iAP mRNA expression, the iAP protein levels were decreased in the inflamed colonic mucosa of children with MC and UC, compared to non-inflamed biopsy samples of MC and controls. The decreased protein levels of iAP in the presence of increased iAP mRNA expression suggest that in the inflamed intestinal mucosa the synthesis of iAP is inhibited posttranscriptionally. The decreased protein synthesis in the inflamed intestine may occur through different pathways. As already mentioned, the activation of reactive oxygen species released during inflammation through ubiquitin-proteasome pathways, leads to increased degradation of proteins and reduction of protein levels.

Examining the colonic mucosa of therapy naive children, I found decreased iAP protein levels, so I assume that the decreased levels of iAP in the inflamed mucous membrane may be related to decreased LPS detoxification and consequently to increased TLR4 activation.

Several animal models have shown the beneficial effect of iAP on intestinal inflammation. The best-known animal model for inflammatory bowel disease is the DSS-induced colitis. It is both macroscopically and microscopically in corresponding to the human colonic inflammation. Ramasamay et al. established DSS-induced intestinal inflammation in WT and iAP KO mice. In KO animals the intestinal inflammation seemed to be more severe, and orally administrated iAP tablets reduced the symptoms of colitis in both groups examined.

Animal studies have confirmed the beneficial effects of iAP tablet on colitis, which is demonstrated also in a human study. Our investigation might have therapeutic consequences, because the long-term treatment of IBD is not solved. The current management of IBD consists essentially of conventional therapy, but in therapy-resistant cases more powerful

biological agents are needed. There are several therapeutic trials to rebalance the microflora, which may contribute to the mucosal healing in IBD.

II. The localization of iAP and TLR4 in the colonic mucosa of children with IBD

Our working group previously showed increased TLR4 mRNA expression and protein levels in the inflamed colonic mucosa of children with IBD. Immunofluorescent staining was performed to examine the localization of iAP and TLR4. Distribution of iAP was clearly seen on the epithelial surface of the terminal ileum and colon as well, and co-localization with TLR4 was detected. The iAP-TLR4 complex formation may strengthen their role in the maintenance of mucosal integrity. Reduced levels of iAP and higher expression of TLR4 may lead to the iAP/TLR4 balance shift, so the mucosa will be less resistant to bacterial LPS, which may result in the reduction of intestinal integrity and maintenance of intestinal inflammation.

The examination of iAP activity may be also a suitable histological method to separate MC and UC. Markers of stool are often analyzed to follow disease activity, relapse or remission, and to track the effectiveness of therapy. The iAP activity was significantly lower in the stool of patients with active UC compared to the control group. Moreover the same parameters were higher in the inactive UC and MC patients, but they showed a significant decrease compared to controls.

III. The alteration of iAP expression in the duodenal mucosa of children with CD

In CD gluten and related proteins induce chronic intestinal inflammation and consequently deterioration of mucosal barrier integrity in genetically susceptible individuals,. The TLR pattern-recognition receptor family is a connecting bridge between the innate and adaptive immune responses. Previously our research group found increased TLR4 mRNA expression and protein levels in the duodenal mucosa of children with newly diagnosed CD. In the peripheral blood of children with newly diagnosed CD the prevalence of TLR4-positive cells was similar to the mucosal findings. These data confirm that innate immunity, especially TLR4 may contribute to pathogenesis of CD. The LPS detoxification activity of iAP plays an essential role in preventing the formation of TLR4-LPS complex, thereby maintaining mucosal barrier integrity.

I first examined the iAP mRNA expression and protein levels in newly diagnosed CD patients

and children with CD maintained on GFD. The iAP mRNA expression was not significantly different in the duodenal mucosa of newly diagnosed and GFD-treated CD children compared to controls. In GFD-treated CD children the iAP mRNA expression showed a slight increase compared to controls, which may be explained by the incomplete recovery of the small bowel mucosa.

The iAP protein levels were significantly decreased in the newly diagnosed, untreated CD patients compared to controls. The decrease of iAP proteins may be related to the decline of iAP enzyme activity. Prasad et al. have shown that the activity of iAP and other brush border-bound enzymes are in correlation with the histological severity of lesions in CD children. One explanation could be that the production of iAP depends on the number of enterocytes and the iAP enzyme could be an indicator of intestinal cell regeneration. Our results suggest that not only the iAP enzyme activity, but the iAP protein levels might be correlated with the severity of CD affecting the intestinal mucosa.

IV. The localization of iAP and TLR4 in the duodenal mucosa of children with CD

Our group previously showed that TLR4 mRNA expression and protein levels are increased in the duodenal mucosa of children with newly diagnosed CD. In addition, the colocalization of iAP and TLR was shown in children with CD. iAP was exclusively localized in the duodenal epithelial surface of the newly diagnosed and GFD-treated CD children, and in the control group as well. The iAP-TLR4 colocalization was intense. The colocalization of iAP and TLR4 may refer also to their functional relationship. It is assumed that the decrease of iAP protein levels and the increase of TLR4 protein levels collectively contribute to the disintegration of intestinal barrier in CD.

Many studies have been published to replace the gluten-free diet in CD with an alternative option of adjuvant therapy. In CD the implementation and maintenance of a gluten-free diet worsens the quality of life and in comparison to normal diet it is more expensive. So a low-risk, more economical and effective treatment is the goal of research. The monotherapeutic application of iAP would be probably not sufficient to replace the gluten-free diet, but with other new therapies used in CD it may contribute to the rapid mucosal regeneration. iAP is able to restore mucosal barrier integrity by its LPS detoxifying activity, preventing the increased and uncontrolled activation of TLR4.

Summarizing my results, the iAP protein levels decreased in inflamed colonic mucosa of children with newly diagnosed MC and UC compared to controls. The iAP protein levels were reduced in the duodenal mucosa of children with newly diagnosed CD in comparison to the control group and children with CD maintained on GFD. The iAP mRNA expression did not change significantly neither in IBD, nor in CD groups studied. The iAP-TLR4 complex formation was also detected in the duodenum, terminal ileum and colon epithelium.

The lower iAP levels may result in decreased LPS-detoxification capacity, and this may contribute to an increased intestinal permeability. According to the literature, it is suggested that the iAP tablets may be effective in mice with DSS-induced colitis and in adult UC patients. Hence the exogenously administrated iAP enzyme might be an adjuvant therapy in active IBD and newly diagnosed CD patients, if controlled studies confirm this hypothesis.

THESES

The most important statements of my Ph.D. thesis are:

(1) First, I showed that the iAP protein levels are reduced in the inflamed colonic mucosa of children with MC and UC, compared to non-inflamed areas from MC and control samples. The iAP mRNA expression showed no significant difference between the studied groups. These results suggest that iAP plays an important role in maintaining the mucosal barrier integrity and the decrease in its protein levels may contribute to the development of IBD as well.

(2) I found that the distribution of iAP was restricted to the epithelial surface of the colonic and terminal ileal mucosa in all groups studied. The colocalization of iAP with TLR4 was intensely stained with dotted-like pattern. This finding is consistent with previous observations of our group, that the expression of TLR4 is increased in the peripheral blood and in the intestinal mucosa as well. The complex formation of iAP-TLR4 may indicate the role of iAP in triggering innate immune responses and LPS detoxification.

(3) I pointed out the decrease in iAP protein levels in newly diagnosed children with CD, compared to children with CD maintained on gluten-free diet and control group. The iAP mRNA expression showed no significant difference between the studied groups. Previously the enzyme activity of iAP was studied in CD and the reduction of iAP enzyme activity in the duodenal mucosa of children with CD was correlated with the severity of histological lesions. This observation and our results suggest that decreased levels of iAP proteins may play a role in the pathogenesis of CD.

(4) I have demonstrated the colocalization of iAP and TLR4 on the surface of duodenal enterocytes and the 3 groups studied showed no difference in staining. Our group previously showed that the prevalence of TLR4 positive cells increased in peripheral blood in children with newly diagnosed CD, while it remained elevated in children with CD maintained on GFD compared to controls. In the duodenal biopsy specimens the TLR4 levels were also increased in newly diagnosed CD and in GFD-treated group. The iAP may contribute to the detoxification of LPS, thereby preventing the LPS-TLR4 complex formation.

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THE LIST OF OWN PUBLICATIONS

Publications in the topic of the thesis

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