Inducible Nitric Oxide synthase in duodenum of children with *Giardia lamblia* infection

Małgorzata Mokrzycka¹, Agnieszka Kolasa², Anita Kosierkiewicz³, Barbara Wiszniewska²

¹Department of Pediatrics, Hematology and Oncology of Children Pomeranian Medical University in Szczecin, Poland
²Department of Histology and Embryology Pomeranian Medical University in Szczecin, Poland
³Department of Pathology Pomeranian Medical University in Szczecin, Poland

Abstract: The investigation were performed on children with *Giardia lamblia* infection, diagnosed on the basis of positive stool tests for Giardia antigen (Elisa) or by microscopical detection of trophozoites in duodenal fluid. In duodenal biopsies morphological studies and immunohistochemical reaction for inducible nitric oxide synthase (iNOS) were performed. The control group was made up of duodenal tissue of children with excluded giardiasis and inflammation of the upper part of gastrointestinal tract. The duodenal biopsies from children without *Giardia lamblia* infection were found to have a high immunoreactivity for iNOS in enterocytes, the cells of intestinal crypts, endothelial cells of vessels and connective tissue cells of lamina propria. In children with giardiasis: in some biopsies the expression of iNOS was as high as in control group, in others was weaker detectable and the shortening of intestinal villi was seen. There were also duodenal biopsies with the lack of immunoreactivity for iNOS, with shorter villi and a large amount of mucus in the intestinal epithelium. Beside of goblet cells, also enterocytes were loaded with mucus. The pathological changes may cause malabsorption and also may have a negative influence on the defense of the intestinal wall against *Giardia lamblia* infection. The different morphological and immunohistochemical results in the duodenum of children with giardiasis can elucidate a variety of clinical symptoms from asymptomatic to severe infection.

Key words: duodenal biopsies, *Giardia lamblia*, iNOS expression

Introduction

Protozoan *Giardia lamblia* is a widespread parasite of the gastrointestinal tract in human as well as domestic and wild animals [1,2]. It mainly inhabits the small intestine but can also live in the gallbladder and pancreatic tracts [3]. The parasite exist in two forms: the infectious endospore cysts, which are resistant to many environmental factors and trophozoites causing clinical symptoms and the disease [4]. Excitement of ingested cysts is due to the low pH level in the stomach as well as elevated pH and proteolytic enzymes in duodenum, whereas excitation requires elevated pH and bile [3]. Trophozoites are able to attach to the microvillous brush border of enterocytes [5] and penetrate crypts but do not invade mucosa [3]. However, there are also investigations indicating that some strains of *Giardia lamblia* disrupt the tight-junctional ZO-1 between intestinal epithelial cells and significantly increase paracellular permeability due to Giardia induced enterocyte apoptosis [6-8]. The interaction between parasites and host shows a number of clinical symptoms. In many individuals, the infection remains asymptomatic whereas other patients exhibit severe symptoms [1,9]. The most important clinical signs of giardiasis include: loss of appetite, abdominal pain, chronic diarrhea, nausea, vomiting and malabsorption. The duration period of disease can vary considerably. In majority of cases giardiasis is a self-limiting process, indicating existence of a host defense against the parasite. One of them is nitric oxide (NO) produced by nitric oxide synthase (NOS). In the digestive tract NO performs many functions including: peristaltic movement, action of sphincters, enlargement of...
the mucosal blood vessels, inhibition of plateletes and leukocyte adhesion and/or aggregation within the vascuclature [10-12]. NO is also involved in the host defense against invading bacteria and parasites.

Nitric oxide – a short-living free radical with biological function, is synthesized from L-arginine through the activation of nitric oxide synthase (NOS). The enzyme exist in three isoforms: endothelial nitric oxide synthase (eNOS), neuronal nitric oxide synthase (nNOS) and inducible nitric oxide synthase (iNOS). Two of them eNOS and nNOS are constitutive calcium-dependent enzymes (cNOS) [11]. Apart from vascular endothelial cells and neuronal cells they are also expressed in other type of cells [13-15]. The third isoform – inducible nitric oxide synthase (iNOS) is calcium-independent and after activation by cytokines and bacterial endotoxins, produces a large amount of NO for an extended period [12,16]. In our former studies we showed iNOS mRNA in a freshly isolated epididymal epithelial cells of control rats [13] and we also showed iNOS immunexpression in the cells of testes and epididymis of rats, without induction with endotoxins and cytokines [17]. The recent findings also revealed the constitutive expression of iNOS in horse testis interstitial cells and in epididymal epithelial cells [14].

In polarized intestinal epithelial cells the stable end products of NO, nitrite and nitrate are detected at the apical side of the cells [18,19]. Because trophozoites remain in the close contact with the epithelial cells in duodenum [20], NO may be a potential host defense against Giardia lamblia.

Therefore the aim of the study was to investigate the expression of iNOS in duodenal biopates from children with giardiasis.

Materials and methods

Patients. The study was performed on duodenal biopates from children, aged 5 – 15 years, hospitalized in the Department of Pediatrics, Hematology and Oncology of Children, Pomeranian Medical University in Szczecin. The patients were admitted to hospital because of chronic abdominal pain and/or chronic diarrhea. Giardiasis was diagnosed in 16 children on the basis of positive stool tests for the Giardia antigen (Crypto/Giardia Duo Strip, Coris Diagnostics, Hematology and Oncology of Children, Pomeranian Medical University in Szczecin; Coris BioConcept, Belgium) or by microscopical detection of trophozoites in duodenal fluid, obtained by aspiration from a naso-duodenal tube.

In all children several tests were performed including routine serum and urine biochemical tests, stool culture, stool sample examination for Giardia antigen, ova of parasites and abdominal ultrasound. Gastroscopy with duodenal biopsy was also done to exclude oesophagitis, ulcer disease or celiac disease.

The control group was made up of duodenal biopates of 8 children without Giardia infection. In 5 biopsies the expression of iNOS was faintly detectable, especially in the control group. In 8 cases the duodenal villi were short and iNOS was faintly detectable, especially in the epithelium of basal part of intestinal villi. There were also areas of lamina propria with negative reaction in intestinal crypts (Fig. 3). The amount and localization of mucin was similar in the above cases to that found in duodenal biopates of children in control groups (Fig. 4).

## Results

Histological examination of duodenal biopsies from 8 children without Giardia infection revealed no pathological changes in the mucosa layer. Therefore this group of 8 children was recognized as a control group. In slides obtained from these biopsies a strong iNOS-reactivity was detected in the basal and particularly in apical side of enterocytes in villi epithelium. Immunexpression of iNOS was also noticed in the cells of intestinal crypts, in connective tissue cells and in the endothelial cells of lamina propria vessels (Fig. 1 A and B). In slides stained with PAS method, a thin layer of mucin was present on the surface of the intestinal epithelium. The goblet cells were filled with mucus (Fig. 2).

In children with Giardia lamblia infection the product of immunohistochemical reaction for iNOS was noticed in the cytoplasm of intestinal epithelial cells, with different intensity. In 5 biopsies the expression of iNOS was on the same level or nearly the same as in control group. In 8 cases the duodenal villi were shorter and iNOS was faintly detectable, especially in the epithelium of basal part of intestinal villi. There were also areas of lamina propria with negative reaction in intestinal crypts (Fig. 3). The amount and localization of mucin was similar in the above cases to that found in duodenal biopates of children in control groups (Fig. 4).
In 3 Giardia-positive duodenal biopsies a notable degree of villi shortening was visible. In these biopsies the immunoperoxidase staining for iNOS both in enterocytes and intestinal crypts was negative (Fig. 5). A trace amount of immunoreactivity for iNOS was sometimes found in connective tissue cells, especially in upper part of villi (Fig. 7,8). In these cases there was a large amount of mucus in intestinal epithelium. Besides goblet cells, mucus was also present in enterocytes (Fig. 6). Numerous lymphoidal cells were localized in lamina propria and epithelium.

**Discussion**

Intestinal epithelial cells are known to be NO-producing cells for affecting parasitic infection [22,23]. However NO generally shows a dual behavior: at physiological concentration, realized through the eNOS (calcium-dependent NOS) regulates house-keeping functions, whereas under the influence of bacteria or parasites an overexpression of iNOS is observed [11]. The intestinal epithelial cells expressed iNOS in a large amount [1]. It has been also shown that the distal small bowel in mice expressed iNOS mRNA constitutively [2]. Also isolated human duodenocytes produce NO constitutively [24]. The intestinal epithelial cells dispose effective NO production, as a potential host defense mechanism against *Giardia lamblia*. Additionally, also fibroblasts and macrophages, cells immediately underlying the epithelium, can produce NO against *Giardia*, via diffusion through the epithelial layer.

In our study of children without giardial infection, the immunoreactivity for iNOS was intensive in enterocytes, largely confined to the apical side of the cells and in duodenal crypts. Immunoreactivity of iNOS was also confirmed in connective tissue cells and endothelial cells of lamina propria vessels. The immunorexpression for iNOS in normal enterocytes and other intestinal cells indicates, that the isoform of the enzyme is expressed in small intestine without inflammation or giardial infection. It should not be unexpected because the intestinal epithelium is always exposed to foreign antigens, including bacteria and their products as well as parasites. In this field our results are in agreement with other authors [22,23].
*Giardia lamblia* was present in the duodenal fluid of 16 children. Among these in 5 biopsies there were no morphological alternations and the intensity of immunoreactivity for iNOS was on the similar level as in control biopptates.

Intestinal epithelial cells-derived NO, is a potential host defense against *Giardia lamblia*. As it was mentioned above, these cells revealed also increased expression of NOS in inducible isoform due to cytokines and microbial products and in constitutive one [1,2,24]. The stable end products of NO, nitrite and nitrate are preferentially detected mainly in the apical side of enterocytes [18,19]. It has been shown that NO has an inhibitory effect on growth and differentiation of *Giardia lamblia* without affecting viability. Therefore it has been found to be cytostatic but not cytotoxic for *Giardia lamblia* trophozoites [18]. Production NO together with other antiangiastial host defense: antimicrobial peptides such as defensins and lactoferrins [25-27], B cells [1,9], mast cells [28] and particularly secretory IgA antibodies [1,29] have an influence on duration of infection and severity of symptoms, leading in the majority of cases to self-limiting of the disease. This is probably the reason of lack of morphological alternations and the intensive immunoreexpression for iNOS, the basic enzyme in synthesis of NO, in the biopptates of 5 children with *Giardia lamblia* infection.

However in biopptates from 8 children shortening of duodenal villi and weaker immunoreactivity for iNOS were detected, especially in the epithelium of basal part of intestinal villi. There were also areas of lamina propria with negative reaction in intestinal crypts. However in three *Giardia*-positive cases the duodenal villi were notable shorter and the immunooexpression of iNOS both in enterocytes and intestinal crypts was negative. A trace amount of immunoreactivity for iNOS was sometimes found in connective tissue cells, especially in upper part of villi. In these 3 cases a large amount of mucus in intestinal epithelium was found. Additionally, enterocytes similarly to goblet cells, also were loaded with mucus. Numerous lymphoidal cells...
Duodenal iNOS in *Giardia lamblia* infection

were localized in the epithelium and in connective tissue villi.

The investigations performed *in vitro* in cultured human intestinal epithelial cells with *Giardia lamblia*, elucidated some mechanisms of NO production in the presence of the parasite in intestineum and the reaction between the pathogen and the host [1]. In spite of potential anti-giardial activity of NO, *Giardia lamblia* has strategies to evade this potential host defense. In co-cultures of human intestinal epithelial cells and *Giardia lamblia* trophozoites, *Giardia lamblia* inhibits the production of epithelial NO by taking up and effective consumption of arginine, the basic substrate in NO production. Arginine is an important source of energy for the parasite [1]. As a by-product of this reaction ornithine is released [30,31], which additionally competitively inhibit arginine uptake by the intestinal epithelium. Both mechanisms reduce the availability of arginine for enterocytes and in this way inhibit NO production. However this findings do not explain the lack of iNOS expression in the biopsies of 3 children with severe morphological alterations and high amount of mucus in the intestinal epithelium.

In the studies performed *in vitro* [18] *Giardia lamblia* infection had no effect on epithelial iNOS expression. It is difficult to say if the same situation takes place *in vivo*. In the normal human airways NO synthesis is due to the continuous expression of iNOS in the airway epithelial cells. However, removal of epithelial cells from the *in vivo* airway environment leads to rapid loss of iNOS expression, which suggests the expression to be depended upon specific condition and or present factors [32]. It is therefore possible that epithelial iNOS expression can be regulated in different pattern, in this case probably due to appearance of mucus in enterocytes.

Intestinal mucins covering the surface of intestinal epithelium are large glycoproteins with high-charge density from sialic acid and sulfate residues as well as protease resistance and water holding capacity. Mucins synthetized by goblet cells can be categorized into two main classes based on their location and structure: membrane bound and secreted forms [33]. Only the latter contribute in the formation of mucus gel. Intestinal mucins are the constituent of luminal barrier function. This is the first line of host defense against enteric pathogens, preventing attachment of them to the mucosal surface. It has been shown that such mucus constituents as glucose and mannose [34], as well as N-acetyl-glucosamine, N-acetyl-galactosamine and fucose [35], inhibit the attachment of *Giardia lamblia* trophozoites to the epithelial cells. Langford et al. [29] demonstrated that antibodies of IgA are required for effective clearance of *Giardia muriis* and *Giardia lamblia* from the murine host by immobilization or detachment of trophozoites from epithelium. IgA is the most abundant immunoglobulin in the mucosal secretion.

In the presence of pathogen in the intestinal lumen the host defence reacts quickly. In response to intestinal microbes mucin secretion is enhanced, and this rapid secretion is thought to provide an important mechanism of protection by eliminating intestinal pathogens. Many enteric microbes and their toxins are known to have a potent secretagogue effect on goblet cells [36]. This can lead to elimination of offending pathogen, because the epithelial surface sloughs off the tip of the villi every 72 hours, together with attached trophozoites.

In 3 *Giardia*-positive duodenal biopsies with large amount of mucus in the intestinal epithelium, the expression of iNOS was negative. The presence of mucus within the cytoplasm of enterocytes, in the same place where intensive reaction for iNOS was commonly confirmed, proves that the cells changed their function. This phenomenon besides of malabsorption may have a negative influence on the defence of the intestinal wall. From the medical history of these children it is known, that the 3 cases of giardiasis were resistant for treatment [unpublished data]. It is difficult to foresee if the stated changes are long-lasting or if they were for a short time. Nevertheless, the different morphological and immunohistochemical state of duodenal lamina propia can elucidate a variety of clinical symptoms from asymptomatic to severe infection in children with giardiasis.

References


