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Alimentary Tract

Patients with mild enteropathy have apoptotic injury of enterocytes similar to that in advanced enteropathy in celiac disease

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ABSTRACT

Background: Severity of villous atrophy in celiac disease (CeD) is the cumulative effect of enterocyte loss and cell regeneration. Gluten-free diet has been shown to benefit even in patients having a positive anti-tissue transglutaminase (tTG) antibody titre and mild enteropathy.
Aim: We explored the balance between mucosal apoptotic enterocyte loss and cell regeneration in mild and advanced enteropathies.
Methods: Duodenal biopsies from patients with mild enteropathy (Marsh grade 0 and 1) ($n = 26$), advanced enteropathy (Marsh grade ≥ 2) ($n = 41$) and control biopsies ($n = 12$) were subjected to immunohistochemical staining for end-apoptotic markers (M30, H2AX); markers of cell death (perforin, annexin V); and cell proliferation (Ki67). Composite *H*-scores based on the intensity and distribution of markers were compared.
Results: End-apoptotic markers and marker of cell death (perforin) were significantly up-regulated in both mild and advanced enteropathies, in comparison to controls; without any difference between mild and advanced enteropathies. Ki67 labelling index was significantly higher in crypts of mild enteropathy, in comparison to controls, suggesting maintained regenerative activity in the former.
Conclusions: Even in patients with mild enteropathy, the rate of apoptosis is similar to those with advanced enteropathy. These findings suggest the necessity of reviewing the existing practice of not treating patients with mild enteropathy.

1. Introduction

Just as the clinical manifestations in celiac disease (CeD) is varied from the asymptomatic to the classical symptoms, the pathological lesions in the intestinal mucosa also vary from mild to advanced enteropathy [1]. Clinical manifestations of the disease often do not appear till the reserves of a particular organ are overwhelmed by the disease process. Hence, overt clinical manifestations in a disease, are generally indicative of an advanced disease state. While a diagnosis of CeD is made once there is advanced enteropathy such as villous atrophy, there are patients with a positive serology but have either no enteropathy or mild enteropathy defined as potential CeD [2,3]. Nutritional deficiencies are described in serology positive patients even without having advanced enteropathy [4,5]. As per the current recommendations, only patients with advanced enteropathy CeD are recommended to be treated with gluten-free diet (GFD) [6–8]. Kurppa et al. had shown in different studies and randomized control trials, the benefit of GFD in patients with mild enteropathy (Marsh 1 and 2) in terms of improvement of clinical symptoms, antibody titre and reduction of mucosal inflammatory activities after institution of GFD in them [9–11]. Subjects with mild enteropathy, who continued with gluten consumption, developed worsening of clinical symptoms and progressive villous abnormalities [10,11]. Such observations raises a question, should not patients having potential CeD be treated GFD?

Villous atrophy is actually a trade-off between the rapidity/severity of enterocyte loss and the enormous regenerative capability of the crypt reserve cells to replace the dying enterocytes [12,13]. While nutritional deficiencies and response to GFD

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have been demonstrated in subjects with potential CeD, the effect of GFD on the enterocyte loss and regeneration has not been studied. We have earlier shown that patients with CeD have a higher rate of apoptosis of epithelial cells in the duodenal mucosa [12]. As the enterocyte apoptosis/cell regeneration appears to be the prime event in CeD, we hypothesized that, apoptosis of enterocytes also occurs in patients with a positive celiac specific serology, but they have mild enteropathy because of the ability of the crypts to match the rate of enterocyte loss secondary to immune mediated apoptosis.

2. Patients and methods

Design: Single centre cross-sectional observational study.

Setting: The study was performed in a tertiary care hospital.

Ethical clearance: The study was approved by the Ethics Committee of our institution and informed written consent was taken for endoscopic examination and duodenal biopsies [*Ref No.: IEC/NO-440/3013 RP-22/2013*].

2.1. Participants

2.1.1. Recruitment of patients with mild and advanced enteropathy

From the database of CeD Clinic at our centre, we retrieved the list of twenty-six patients suspected to have CeD, with positive serum anti-tissue transglutaminase antibody (anti-tTG Ab) (mean anti-tTG Ab titre was 8.1 ± 5.6 times the cut-off for a positive test), but having no/mild enteropathy (potential CeD) [modified Marsh grades 0 and 1]. The patients with Marsh 0, was also included as mild enteropathy, first for simplicity and also due to that fact that, a few biopsies with Marsh 0 showed some histological changes as crypt hyperplasia with increased mucosal inflammatory cell infiltrate. The stool tests for parasitic infection such as *Giardia lamblia*, and *Cryptosporadium* were negative in them. We also randomly selected from our database, 41 seropositive patients with confirmed CeD (with average anti-tTG antibody titre 7.1 ± 6.2 times the cut-off for a positive test) having advanced enteropathy [villous abnormalities of modified Marsh grades 2, grade 3a, 3b and 3c].

2.1.2. Recruitment of controls

Additionally, we recruited 12 subjects with functional dyspepsia who underwent upper gastrointestinal endoscopic examination for their diagnosis. They did not have diarrhoea, anaemia and had normal serum anti-tTG Ab titre.

2.1.3. Characteristics of the subjects

The demographic details, clinical parameters, serological and histological data of all the subjects were retrieved. The serological test for CeD was done using anti-tTG Ab ELISA [AESKU Diagnostics (Wendelsheim, Germany)] diagnostic kit. The test was done as per manufacturer's instructions and positive and negative controls were used every time. An anti-tTG titre of >18 U/ml was considered positive and a titre between 12 and 18 U/ml was considered as borderline. The serological test was repeated if the titre of anti-tTG antibody was found to be borderline for the confirmation of the titre. Patients only with a positive anti-tTG Ab titre were included in this study. The results of anti-tTG Ab titre were expressed as fold rise above the cut-off value (by calculating the ratio of observed anti-tTG Ab titre, divided by the cut-off value of a positive test) and was compared amongst different study groups [Table 1]. The severity of villous abnormalities was graded by using the modified Marsh classification system [3]. The diagnosis of CeD was made based on the modified European Society of Paediatric Gastroenterology, Hepatology and Nutrition criteria [6].

2.2. Investigations

2.2.1. Biopsy characteristics

Four endoscopic duodenal mucosal biopsies from each patient and controls, were included for study. The biopsies were mounted with the mucosal surface "up" on filter paper, processed routinely, and embedded in paraffin. Haematoxylin and Eosin stained sections were evaluated under light microscope. If at least 3–4 longitudinal crypts were found perpendicularly arranged on the muscularis mucosae in a row, the biopsy was considered to be oriented satisfactorily. Where the initial sections did not show satisfactory orientation, multiple step sections, up to 50–60, were examined as per our standard protocol for reporting of intestinal mucosal biopsies. The biopsies which were not properly oriented were excluded from the study. Histological characteristics were reported as per modified Marsh Oberhuber classification system [3].

2.2.2. Immunohistochemistry for markers of apoptotic and regenerative activities in the duodenal mucosal biopsies

From the paraffin embedded blocks, 4–5 μm sections on poly-L-lysine coated slides were processed for immunohistochemistry (IHC). All these slides were subjected to IHC staining for: (i) *end apoptosis marker proteins*: M30 (Santa Cruz Biotech Inc, Europe; 1:500), and H2AX (Santa Cruz Biotech Inc, Europe; 1:1000); (ii) *markers of cell death*: Perforin (Santa Cruz Biotech Inc, Europe; 1:400), and AnnexinV (AbCam, CA, USA; 1:1000)) and (iii) *marker for cell proliferation*: Ki67 (Spring Bioscience, CA, USA; 1:500), using primary monoclonal antibodies. Standard overnight IHC staining protocol was followed in this study. Universal secondary antibody (CRF anti-polyvalent HRP polymer, ScyTek Laboratories, Logan, USA) was used and the reaction was developed by using 3,3′-diaminobenzidine. Appropriate positive controls such as reactive lymph nodes and squamous cell carcinoma (for M30 stain) were used. Negative controls were included in each batch by skipping the primary antibody. The groups of apoptotic markers were chosen based on our previous experience [12,13].

Interpretation of IHC stain was done in respect to the distribution and the intensity of the stains, both in the crypts and the villi of mucosal biopsies separately. The distribution of marker staining was graded as follows: grade 0: no staining to <10% mucosal area positivity; grade 1: 11–20% area positivity, grade 2: 21–40% area

Table 1
Clinical characteristics and serological data in patients.

Characteristics	Controls	Villous abnormality (modified Marsh grade 0–1)	Villous abnormality (modified Marsh grade 2–3)
Age (yrs), mean ± SD	32.2 ± 12.8	22.5 ± 7.8	27.8 ± 13.8
Duration of symptoms (yrs)	Not applicable	1.0 ± 1.6	1.4 ± 2.3
No of patients having diarrhoea	Not applicable	16	23
Haemoglobin level [mean ± SD] in gm/dL	12.2 ± 2.6	9.1 ± 3.3	9.3 ± 3.2
Anti tTg Ab expressed as fold- rise, worked out by calculating the ratio of the observed value with cutoff value [mean ± SD]	Not applicable	8.1 ± 5.6	7.1 ± 6.2

Ab, antibody; N/A, not applicable; WNL, within normal limit.

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