FOXP3 Expression in Duodenal Mucosa in Pediatric Patients with Celiac Disease

Eli Brazowski a Shlomi Cohen b Ayala Yaron b Irina Filip a Avi Eisenthal a

a Pathology Institute, and b Pediatric Gastroenterology Institute, Tel-Aviv Sourasky Medical Center, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel

Introduction

Celiac disease is among the most common inherited diseases with a worldwide prevalence of almost 1% of the population [1, 2]. It is a small intestine inflammatory disorder that causes a permanent food intolerance to ingested gluten in genetically susceptible individuals. Although it shares many immunologic features with inflammatory bowel disease, celiac disease is uniquely characterized by a defined trigger of gluten proteins, such as gliadin, the required presence of HLA-DQ2 or HLA-DQ8, and the generation of circulating autoantibodies to the enzyme transglutaminase type 2 (TG2). The exact pathogenesis of celiac disease remains unclear. Several studies have shown that TG2 catalyzes cross-linking or deamidation of glutamine present in gliadin [3, 4], which apparently increases their affinity to HLA-DQ2 or HLA-DQ8 [5]. This complex of gliadin with the HLA class II molecules generates an increased number of intraepithelial lymphocytes, elevation of interleukin (IL)-15 [6] and activation of T-helper 1 CD4+ T cells. Intestinal mucosal inflammation leads to increased numbers of CD8+ cytotoxic lym-
phocytes that infiltrate into the epithelial mucosa [7], production of interferon-γ, crypt cell hyperproliferation, villous atrophy, mucosal damage malabsorption, numerous secondary symptoms and autoimmune diseases.

Regulatory T cells (Tregs) are a particular subset of CD4 T cells with a constitutively high density of CD25-CD25^{high} [8] as well as of the glucocorticoid-induced tumor necrosis factor receptor. The best indicator of Treg function is the intracellular expression of FOXP3 [9], a forkhead family transcription factor, as a critical regulator of Treg development, function and homeostasis [10, 11]. Genetic mutations in the gene encoding FOXP3 have been identified in both mice and humans [12, 13]. Tregs aid in the maintenance of self-tolerance by suppressing the proliferation and cytokine production of autoreactive lymphocytes, apparently by cell-to-cell contact [14, 15] after specific T-cell receptor activation by self-antigens in the presence of IL-2 [16]. In addition, these cells exhibit regulatory function in human and murine models of autoimmunity [9, 17] as well as in cancer [18]. Tregs are regulated by cytokines, such as IL-15 [19], which maintain their optimal regulatory function in a phosphatidylinositol 3-kinase-dependent manner [20] and induce Treg proliferation through interaction with their T-cell receptor [21].

Since one of the events in the pathogenesis of celiac disease is the activation and expansion of lymphocytes by cytokines [6, 22, 23], some of which regulate Tregs, we employed immunohistochemistry methods to examine the presence of FOXP3^{+} and CD8^{+} cells in biopsies of the descending duodenum mucosa from celiac patients. Our results indicated that unlike CD8^{+} cells, the number of FOXP3^{+} cells in the lamina propria (LP) of patients with celiac disease is related to the Marsh classification [24].

**Patients and Methods**

**Patients**

Sixty-one children with untreated celiac disease (25 males and 36 females, mean age 5.7 ± 3.9 years, range 1–15) were enrolled in the present study. These children were referred to the gastroenterology department because of symptoms that included diarrhea, iron deficiency, abdominal pain and weight loss, or because of being serologically positive for anti-endomysial antibodies. They all underwent upper gastrointestinal endoscopy, and biopsy specimens were obtained from the descending duodenum. The control, non-celiac (NC) group included 21 dyspeptic patients (9 males, 12 females; mean age 10.7 ± 4.0 years, range 2–16) who were anti-endomysial antibody negative, had no history of celiac disease in the family, were not on medication and were free of other gastrointestinal disease and food intolerance.

**Histological Assessment**

Histological assessment of celiac disease was performed on formalin-fixed paraffin-embedded tissue sections after staining with hematoxylin-eosin, based on the following parameters: villous atrophy, increased number of intraepithelial lymphocytes, crypt hyperplasia and lymphoplasmacellular infiltrate in the LP. Histological lesions were classified as follows according to the Marsh classification [24] that consists of a 4-stage system with 40 intraepithelial lymphocytes per 100 epithelial cells as the normal upper limit: type I (Marsh I, M1), infiltrate lesion, characterized by intraepithelial lymphocytosis and normal villous architecture of duodenal mucosa; type II (M2), hyperplasia lesion, characterized by intraepithelial lymphocytosis and crypt hyperplasia with a normal villous architecture; type III (M3), destructive lesion, characterized by intraepithelial lymphocytosis, crypt hyperplasia and villous atrophy, and type IV (M4), hyperplastic lesion, characterized by a normal intraepithelial lymphocyte count, normal crypt length and villous atrophy. All celiac cases in our study were classified as M1, M2 or M3.

**Antibodies**

Mouse anti-human FOXP3 monoclonal antibody (Cat. No. AB20034-250, Abcam, UK) diluted 1:25 and mouse anti-human CD8 monoclonal antibody (Cat. No. RM-9116-S, Neomarkers, Fremont, Calif., USA) diluted 1:100 were employed for immunohistostaining [25].

**Paraffin-embedded** tissue sections of 3–5 μm underwent de-paraffinization, heat-induced antigen retrieval at controlled temperature using CC1 buffer (Cat. No. 950-124, Ventana, Tucson, Ariz., USA), followed by incubation for 32 min with either 1:25 diluted FOXP3 or 1:100 diluted CD8 antibodies. Thereafter, tissue sections were incubated with an I-View DAB detection kit (Cat. No. 760-091, Ventana), according to the manufacturer’s instructions. For double staining, FOXP3 was applied by means of a DAB detection kit followed by an AEC detection kit (Cat. No. 760-020, Ventana) for CD8^{+} cells. FOXP3^{+} cells were identified by dark brown nuclear staining and CD8^{+} lymphocytes by red cytoplasm staining. All staining steps were performed at a controlled temperature of 42°C by an automated staining device (Benchmark XT, Ventana). Stained tissues developed a dark brown staining which was further visualized by counterstaining with Gill’s hematoxylin (Cat. No. 1.05174.2500, Merck, Darmstadt, Germany).

**Microscopic Evaluation of Stained Tissue Sections**

Tissues were stained with both anti-FOXP3 and anti-CD8 antibody. All tissue sections were examined independently by 2 pathology experts in a blind fashion, unaware of the patient’s name and group assignment. The numbers of positively stained mononuclear cells were counted systematically under a light microscope at ×400 magnification. The positive cells in 5 different fields were counted, both in the surface epithelial (SE) mucosa and in the LP mucosa. Cell density was expressed as the mean number of positively stained specimens as scored by the 2 pathology experts. There was a <10% discrepancy in the mean numbers of positive cells for all cases between the examiners. For anti-endomysial (TG2) antibody measurement, TG2 serum levels of each celiac study patient were determined by an enzyme-linked immunosorbent assay, as previously described [26].
Statistical Analyses

Means and standard deviations were calculated for 3 parameters: CD8-LP, FOXP3 and CD8-SE. The study includes 82 patients who could be divided into 4 groups according to their histological grade (i.e. 0-NC group, as well as groups 1, 2 and 3). The Pearson correlation coefficient was calculated between FOXP3 and TG2 for 46 patients with histological grades 1, 2 and 3. The association between FOXP3 and histological grade was evaluated by Spearman’s correlation coefficient. The multiple comparison adjustment method of Tukey-Kramer was employed for pairwise comparisons between groups. The parameters were dichotomized as follows: FOXP3 (0: <15, 1: ≥15), TG2 (0: <40, 1: ≥40) and histological grade (0, 1+). Fisher’s exact test was then used to examine a possible relationship between FOXP3 and both TG2 and histological grade (as binary parameters). The SAS for Windows version 9.2 was used for the analysis.

Results

Correlation between the Presence of FOXP3-Positive Cells in the LP Mucosa and the Histological Grade

As shown in figure 1, FOXP3+ cells were exclusively located in the LP mucosa, while no positive cells were seen in the SE mucosa. Analyzing each individual biopsy for both the number of FOXP3+ cells and the histological Marsh grade revealed a significant positive linear relationship between these 2 parameters (r = 0.3149, p < 0.0001; fig. 2). By employing a cutoff point of 15 for low versus high FOXP3+ cell number, 20/21 (95%) and 7/8 (88%) of the biopsies from NC and M1 patients, respectively, showed a mean of <15 FOXP3+ cells compared with 3/22 (14%) and 4/31 (13%) in M2 and M3 biopsies, respectively (p < 0.0001, Fisher’s exact test). In addition, the mean number of FOXP3+ cells was 8.9 ± 1.1 (SEM), 6.8 ± 2.4, 31.1 ± 2.8 and 24.5 ± 2.6 for the NC, M1, M2 and M3 groups, respectively. The increase in FOXP3+ cells in the M2 and M3 groups was 357 (p < 0.0001) and 200% (p = 0.0010), respectively, compared with the M1 group, and 260 and 136% (p < 0.0001), respectively, compared with the NC group. There was no significant difference in the increase in FOXP3+ cells between the NC and M1 (p = 0.9597) and between the M2 and M3 groups (p = 0.3749) (fig. 2).

Correlation between FOXP3+ Cells in the LP and TG2 Serum Levels

Since TG2 levels are known to be elevated in celiac patients [4], we further examined a possible correlation between TG2 serum levels and the number of FOXP3+ cells in the tissue LP. The correlation between tissue FOXP3+ cells and serum TG2 levels (r = 0.3498, p = 0.017) was significant (fig. 3). When analyzing the data with a cutoff point of 15 for FOXP3+ cells and 40 U/ml for TG2, 92% of cases with FOXP3+ cells >15 showed TG2 serum levels >40 U/ml, whereas TG2 serum levels were <40 U/ml in 60% of cases with FOXP3+ cells <15 (p = 0.0014).
Fig. 2. Correlation between the histological grade (Marsh) and FOXP3+ cells in the epithelial LP. The mean ± SEM is given. 0 = NC group. Multiple pairwise comparison between NC and M1 (p = 0.9597), between NC and M2 (p < 0.0001), between NC and M3 (p < 0.0001), between M1 and M2 (p < 0.0001), between M1 and M3 (p = 0.0010), and between M2 and M3 (p = 0.3749). Spearman correlation coefficients show a positive correlation between FOXP3+ cells and histological grade (r = 0.5608, p < 0.0001). We used a cutoff point of 15: FOXP3+ cells <15 in 27/29 cases in the NC and M1 groups and in 7/53 in the M2 and M3 groups. There is a significant relationship (p < 0.0001, Fisher's exact test) between the histological grade and the number of FOXP3+ cells.

Fig. 3. Correlation between mucosa FOXP3+ cells and serum TG2 levels. Pearson correlation coefficients showed a significant correlation (r = 0.3498, p = 0.017) between FOXP3+ cells and serum TG2. We used a cutoff point of 15 FOXP3+ cells and 40 U/ml TG2: there is a significant relationship (p = 0.0014, Fisher's exact test) between these parameters.

Fig. 4. Presence of CD8+ cells in the epithelial mucosa of celiac patients. The numbers of positively stained mononuclear cells were counted at ×400 magnification. a NC patients. b M1 celiac patients. c M2 celiac patients. d M3 celiac patients.
Presence of CD8+ Cells in the Descending Duodenum Mucosa of Celiac Patients

Having demonstrated an increase in the numbers of FOXP3+ cells in the LP mucosa of M2 and M3 biopsies, we tested whether other lymphoid populations were affected by the disease. To that end, we analyzed the number of CD8+ cells which were shown to be affected in the advanced stages of celiac disease [7]. As shown in figure 4, unlike FOXP3+ cells, CD8+ lymphocytes were present in both the LP and SE mucosa. In addition, nuclear staining of FOXP3+ cells using a double-staining procedure for both FOXP3 and CD8 molecules revealed that these cells were distinct from the cytoplasm-stained CD8+ lymphocytes (fig. 5). By plotting the number of CD8+ cells in the LP mucosa versus the histological Marsh grade for each individual biopsy, the mean number of CD8+ cells in LP was 37.3 ± 2.8, 38.6 ± 8.6, 33.7 ± 3.4 and 47.3 ± 3.2 in the NC, M1, M2 and M3 groups, respectively, with the differences between M2 and M3 reaching a level of significance (p = 0.0180; fig. 6). There were no significant differences in the number of CD8+ cells between the NC (101.0 ± 10.1), M1 (97.4 ± 30), M2 (82.1 ± 17.1) and M3 groups (105.1 ± 7.1) (fig. 7).
Discussion

Celiac disease is characterized by abnormalities in both cell-mediated and humoral immunity following the accumulation of gluten peptides, with the activation of T cells in the epithelial mucosa as one of the manifestations of the disease pathogenesis [7]. In our study, we evaluated the presence of 2 lymphoid populations, CD8+ and FOXP3+ cells, in biopsies taken from the descending duodenum of celiac patients at the M1, M2 and M3 stages of the disease as well as from NC patients. FOXP3+ cells were restricted to the LP epithelial mucosa with a significant positive linear relationship between the number of FOXP3+ cells and the histological Marsh grade (r = 0.3149, p < 0.0001; fig. 2). Using a cutoff point of 15 to differentiate between high and low levels of FOXP3+ cells, it emerged that the mean number of FOXP3+ cells was <15 in 95% of NC and in 88% of M1 biopsies, respectively, compared with 14% of M2 and 13% of M3 biopsies (fig. 2). The mean number of FOXP3+ cells in M2 and M3 biopsies was significantly higher (p < 0.0001) by up to 357 and 200%, respectively, when compared with the mean number of M1, and by 260 and 136% when compared with the NC biopsies. Similarly, there was a significant positive linear relationship between the number of tissue FOXP3+ cells and TG2 serum levels (r = 0.3498, p = 0.017; fig. 3).

The mechanisms regulating the increase in FOXP3+ Treg numbers in M2 and M3 stages of celiac disease, which are similar to those described by others for salivary gland tissue of primary Sjögren’s syndrome [27], are not fully understood. It is one possibility that there is a general increase in lymphocyte activation and expansion which is regulated by proinflammatory cytokines during advanced stages of celiac disease [28]. However, our current results refute this possibility since the number of CD8+ cells in the LP mucosa of M2 biopsies was similar to the number of cells in M1 biopsies (fig. 6), although the number of FOXP3+ cells was substantially increased (by 357%). Interestingly, while the number of FOXP3+ cells in the LP mucosa in M3 biopsies (24.5 ± 2.6) was lower than in M2 biopsies (31.1 ± 2.8), the number of CD8+ cells was significantly higher (47.3 ± 3.2; p < 0.05) in M3 compared with M2 biopsies (33.7 ± 3.4). This observation points towards the possibility that FOXP3+ cells regulate the number of CD8+ cells in M2 biopsies, as has been shown for autoreactive lymphocytes [14, 15]. This FOXP3 regulation of CD8+ cells could be carried out by cell-to-cell contact [29] or by the release of selected cytokines, such as transforming growth factor-β [30]. Others have shown an increase in a CD8+ subpopulation with the CD8CD25 FOXP3 phenotype which was endowed with the ability to inhibit CD4+ self-reactive T-cell proliferation and interferon-γ secretion in multiple sclerosis [31]. Although that study had been carried out in culture, the possibility that regulatory lymphocytes with both the CD8 and FOXP3 phenotype were present in the epithelial mucosa of celiac biopsies could not be excluded. Studies aiming to reveal the presence of various Treg populations in the epithelial mucosa of celiac patients are currently underway.

Another difference between CD8+ and FOXP3+ cells was the restriction of FOXP3+ cells to the LP mucosa, similar to CD4+ lymphocytes (unpublished results), while CD8+ cells were present in both the SE and LP mucosa in all the investigated mucosal damage severity grades. In addition, by using a double immunostaining procedure, FOXP3+ cells were shown to be distinct from CD8+ lymphocytes (fig. 5). This difference could be attributed to the pre-existence of relatively high numbers of CD8+ cells in the SE mucosa compared with their numbers in the LP mucosa (fig. 5–7), which might spatially interfere with the expansion or migration of FOXP3+ cells into this area. Alternatively, although not mutually exclusive, cytokines, such as IL-10 [32] and IL-15 [33], which selectively activate Tregs, could preferentially be secreted in the LP mucosa, thus inducing FOXP3+ cell expansion. Studies designed to reveal the presence of the described cytokines in the epithelial mucosa of celiac patients are also currently underway.

Finally, our findings in the present study demonstrated a significant increase in FOXP3+ cells in the LP epithelial mucosa in biopsies from celiac patients, which correlated with both the histological Marsh grade and serum TG2 levels. Further studies designed to characterize the activity of these cells in celiac and in other inflammatory bowel diseases are warranted. Their results will enable us to understand the significance of these cells in celiac disease.

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References


