Duodenal mucosa FOXP3 expression in different etiologies of lymphocytic duodenosis

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Summary. Background/aims. In celiac disease there is an increase of lymphocytes expressing FOXP3 in the intestinal mucosa associated with varying degrees of villous atrophy. Our aim was to evaluate FOXP3 expression in duodenal mucosa with lymphocytic enteritis according to aetiology and correlation with lymphocytes T-γδ.

Methods. We compared three adult patient groups suffering lymphocytic enteritis: celiacs following a gluten-free diet (n=12), first-degree relatives of celiac patients with genetic risks (n=14) and patients with functional dyspepsia (n=14), along with a control group not suffering from duodenal enteritis (n=16). The population of duodenal lymphocytes was analysed by immunohistochemistry assays for CD3+ characterisation and FOXP3 expression. Quantification of lymphocytes T-γδ in duodenal mucosa was performed by flow cytometry in fresh tissue samples.

Results. Presence of lymphocytes T-γδ was significantly higher in the group of celiac individuals compared to the group of relatives of these individuals (37.44 vs 5.52; p<0.0001) and patients with functional dyspepsia (37.44 vs 11.76; p=0.008). FOXP3 expression was also significantly higher in the celiac group than in the groups of relatives (18.85 vs 6.31; p=0.001) and functional dyspepsia patients (18.85 vs 7.61; p=0.023). The proportion of lymphocytes T-γδ and FOXP3-expressing lymphocytes was similar in the control group to that in the relatives or functional dyspepsia groups.

Conclusions. Lymphocytic enteritis associated to celiac disease shows an increase of FOXP3 expression and lymphocytes T-γδ that is not detected in other etiologies of enteritis.

Key words: Lymphocytic enteritis, Celiac disease, Gluten sensitivity, FOXP3, Flow-cytometry

Introduction

Celiac disease (CD) is an enteropathy sensitive to gluten that affects individuals with genetic predisposition. It is deemed as a multifactorial disorder with a wide range of symptoms and some of the intestinal mucosa disturbances include lymphocytic enteritis and villous atrophy (Vaquero et al., 2015).

Regulatory T cells (Treg) constitute a CD4 T lymphocyte sub-type with presence of CD25+ marker. The best indicator of Treg cells is the intracellular expression of FOXP3 (Factor Forehead Box P3), a transcription factor essential for their production and for their regulatory function (Sakaguchi et al., 1995). Treg cells are indispensable for auto-tolerance control and act on other T lymphocyte populations repressing proliferation and inducing cytokine production in response to activated CD4 T Lymphocytes (Fehervari and Sakaguchi, 2004). Mutations in the FOXP3 coding gene are identified in humans and murine models that result in a decrease in the number and function of Tregs, and this is associated with manifestation of different neoplasia and autoimmune processes (Bennett et al., 2001).
Duodenal FOXP3 expression

Activation and proliferation of CD4 T lymphocytes and Treg cells mediated by cytokines occur during manifestation of CD (De Nitto et al., 2009). In normal conditions, cells expressing FOXP3 represent only a small percentage of lymphocytes in the mucosa. Inflammatory disorders with high infiltration of lymphocytes in the mucosa have a tendency to have an increased proportion of FOXP3-expressing cells (Villanacci et al., 2011). This increase is also observed during active CD, although after following a gluten-free diet and recovering the villous architecture, FOXP3 proportion is reduced. Possibly, CD could be linked to a dysfunction of the regulatory gene of Treg cells in the small intestine. This finding of a regulatory gene dysfunction in peripheral blood cells in active CD highlights a potential role of a general defect in immune tolerance in the disease.

Lymphocytic enteritis is a damage evident not only in CD, but is also present in other different processes, sometimes challenging the establishment of differential diagnostics (Rosinach et al., 2012). In these cases, identification of FOXP3-expressing lymphocytes could help to determine the cause of increase in intraepithelial lymphocytes. Our aim is to evaluate the expression of FOXP3 in the duodenal mucosa in patients with lymphocytic enteritis of diverse etiologies and establish a correlation with the number of duodenal lymphocytes T-γδ analysed by flow cytometry.

Material and methods

Patients

Distinct groups of patients with lymphocytic enteritis were selected for assessment: i) Twelve adult celiac patients (average age 32.6±14.5 years; 58.3% female) that were diagnosed based on ESPGHAN criteria and followed a gluten-free diet for more than two years. These patients showed increased intraepithelial lymphocytes after performing a control duodenal biopsy for assessing recovery of the intestinal mucosa. At the time of the biopsy all the celiac patients showed normal anti-transglutaminase (ATG) antibody levels (<3 UI/ml); ii) Fourteen first-degree relatives (FDR) of celiac patients (average age 42.2±5.5 years; 64.3% female) that were prone to genotype risk for CD (HLA DQ-2 or HLA DQ-8) and had negligible levels of ATG antibody (<3 UI/ml); iii) Fourteen individuals with functional dyspepsia (average age 38.9±11 years; 64.3% female) that were diagnosed based on ROMA III criteria with absence of risk haplotypes for CD and presenting negative serology; iv) A control group of 16 individuals (average age 42.7±9.3 years; 43.8% female) with no histological alterations observed in duodenal biopsies, no family history of CD and risk haplotype, and negative celiac serology. This last group of control individuals was also studied in gastroesophageal reflux cases.

Our ethical committee approved the study, and all the participants signed a written informed consent.

Histological analysis

Histological analysis of celiac disease was performed using formalin-fixed celiac disease tissue sections after eosin-hematoxilin staining based on the following parameters: villous atrophy, increased number of intraepithelial lymphocytes and crypt hyperplasia. Histological damage was rated according to Marsh-Oberhuber classification (Oberhuber et al., 1999). Diagnostics of lymphocytic enteritis was established in cases with >25 lymphocytes/100 enterocytes in the immunohistochemical study of duodenal biopsy.

Antibodies

FOXP3 anti-human mouse monoclonal antibodies (dilution 1:25) and anti-human mouse CD3 monoclonal antibodies were used for immunohistochemistry assay (Cat. No. AB20034-250, Abcam, UK).

Immunohistochemistry

Paraffin embedded tissue sections of 3-5 μm thickness were subjected to de-paraffin process and antigen recovery was induced by heat at constant temperature using CC1 buffer (Cat. No. 950-124, Ventana, Tucson, Ariz, EE.UU.). The sections were incubated with anti-FOXP3 antibody (1:40 dilution) for 32 minutes. Next, tissue sections were incubated with I-View DAB detection kit (Cat. No. 760-091, Ventana) according to the manufacturer’s protocol.

All staining incubations were performed at a constant temperature of 42°C using an automated staining instrument (BenchmarkXT, Ventana). The stained tissue showed a dark brown colour that was further visualised by contra-staining with Gill hematoxilin (Cat. No. 1.05174.2500, Merck, Darmstadt, Germany). The number of positive FOXP3 lymphocytes stained were counted systematically under a light microscope at x400 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 pathologist (M.H.).

Flow cytometry

Flow cytometry was performed with fresh biopsies as previously described. This assay detects the percentage of T-lymphocyte receptor (TCR) γδ+ and CD3+ lymphocytes. Percentage values higher than 13% for TCR γδ+ lymphocytes were considered to be associated with CD.

Statistical analysis

The categorical variables were expressed by number and percentage, whereas quantitative variables were denoted by median and standard deviation (SD). To
compare categorical variables, Chi-square test with Yates correction was applied wherever necessary. To compare quantitative variables the U test of Mann-Whitney and Kruskal-Wallis tests were employed as the variables showed a deviation from the normal curve. To calculate the degree of linear association between two quantitative variables, the Spearman coefficient for ordinal correlation was used. Values of p<0.05 were taken as significant statistical values by two-tail analysis. Bonferroni correction was applied to “post-hoc” analysis.

Results

The main epidemiological characteristics of the different study groups are described in Table 1. All groups, except the control group, showed an increase of intraepithelial lymphocytes defined by more than 25 CD3 lymphocytes per 100 enterocytes.

As observed in Fig. 1, levels of FOXP3-expressing cells changed depending on the distinct groups analysed. The number of FOXP3 lymphocytes localised to the lamina propria of duodenal biopsies was significantly higher (18.9±SD 11.9) for celiac individuals with lymphocytic enteritis following gluten-free diet (GFD) compared to other groups (p=0.005; Kruskall-Wallis test). However, the levels of FOXP3-expressing cells in the control group without lymphocytic enteritis (8.5±SD 2.5) were similar to the levels obtained in individuals with lymphocytic enteritis associated with functional digestive pathology (7.6±SD 2.2) and with FDR (6.4±SD 2.5); p>0.05 for all the paired comparisons (Mann-Whitney U test with Bonferroni correction applied). An example of the FOXP3 staining in each of the groups analysed is represented in Fig. 2.

The percentage of lymphocytes T-γδ in duodenal biopsies determined by flow cytometry was also variable depending on the study groups. The celiac individuals showed 37.4% TCR γδ+ lymphocytes, whereas individuals with functional dyspepsia, the FPG group and the control group had 11.8%, 5.5% and 5.3% respectively (p<0.001; Chi-square test with Yates correction), as shown in Fig. 3. A representation of flow cytometry analysis of IEL lymphocytes from a representative non-celiac patient and a celiac patient are shown in the Fig. 4.

We analysed the relation between the number of FOXP3-expressing cells and lymphocytes T-γδ among all the samples and groups (n=56). We observed a positive linear correlation, which was statistically significant between both variables (r=0.752, p<0.001, Spearman test) (Fig. 5).

Discussion

CD presents alterations in innate and adaptive immune systems that ultimately cause lesions in the duodenal mucosa. The increase in intraepithelial lymphocytes presents a challenge for diagnosis since it is not a pathognomonic lesion of CD and also diverse circumstances can trigger this increase (e.g., *Helicobacter pylori* infection, lactose intolerance, and anti-inflammatory drug consumption) (Rosinach et al., 2012). In our study, identification of cells expressing FOXP3 allows us to differentiate, within the context of lymphocytic enteritis, individuals with CD from individuals with other diseases (e.g., patients infected with *H. pylori*). This presents a potential diagnostic tool that directly correlates with levels of γδ T lymphocytes.

Other studies have observed the relation between the increase in FOXP3 expression in duodenal mucosa layer and active CD with villous atrophy (Brazowski et al., 2010). However, in our research we observed a connection with inactive CD (with GFD) showing an increase of intraepithelial lymphocytes compared to other asymptomatic individual groups; namely, individuals with functional digestive pathology or individuals at high risk of developing CD as they are FDR of celiac patients (Vaquero et al., 2014).

An increase in FOXP3 level suggests the

| Table 1. Main characteristics of the different study groups. |
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                | FDR             | GFD-CD          | FD              | Control         |
| (n=14)         | (n=12)          | (n=14)          | (n=16)          |                 |
| Age (years)    | 42.2±5.5        | 32.6±14.5       | 38.9±11         | 42.7±9.3        | 0.08 |
| Female (%)     | 64.3%           | 58.3%           | 64.3%           | 43.8%           | 0.62 |
| IELs           | 32.6±10.4       | 35.3±9.8        | 32.4±5.2        | 18.1±7.9        | <0.001 |
| *Helicobacter pylori* (%) | 61.5%           | 42.9%           | 53.8%           | 30.8%           | 0.431 |

involvement of T regulatory cells in CD pathogenesis (Vorobjova et al., 2015), and this increase may be associated with some defect in the regulatory gene function of T cells in the small intestine (van Leeuwen et al., 2013). Finding a malfunction regulatory factor in peripheral blood cells highlights the possible role of general defects in immune tolerance in CD (Granzotto et al., 2009). Regulatory T cells are essential for the

Fig. 2. Presence of stained FOXP3+ cells in the epithelial mucosa of the study groups. A. First-degree relatives. B. Celiac patients with lymphocytic enteritis. C. Functional dyspepsia patients. D. Control group without lymphocytic enteritis. x 200.
maintenance of immune homeostasis, application of immune tolerance to self-antigens, and thereby prevent the occurrence of autoimmune phenomena (Barnes and Powrie, 2009).

Previous studies demonstrated the relation between the role of the anti-transglutaminase antibodies (ATG) in peripheral blood and the number of FOXP3 lymphocytes in the intestinal mucosa (Frisullo et al., 2009). However, in our study celiac patients that were following GFD over a prolonged period of time had negative antibody levels. Although GFD reversed the villous atrophy that was diagnosed, the patients still had an increase of lymphocytes in the duodenal biopsy. The incapability of the duodenal mucosa to completely recover is a known fact in adult celiac individuals following GFD (Pekki et al., 2015). The persistence of duodenal inflammation may be related to the inadvertent ingestion of small amounts of gluten and/or persistence of autoimmune mechanisms present in CD. We observed that this increase in lymphocytes is due to a significant expression of FOXP3.

**Duodenal FOXP3 expression**

![Graph showing percentage of lymphocytes T-γδ in duodenal biopsies determined by flow cytometry in the four study groups: FDR: First-degree relatives of CD patients, GFD-CD: Celiac patients on gluten-free diet, FD: Functional Dyspepsia.](image)

**Fig. 3.** Percentage of lymphocytes T-γδ in duodenal biopsies determined by flow cytometry in the four study groups: FDR: First-degree relatives of CD patients, GFD-CD: Celiac patients on gluten-free diet, FD: Functional Dyspepsia.

**Fig. 4.** Flow cytometry Intraepithelial lymphocytes analysis algorithm. Flow cytometry analysis of intraepithelial lymphocytes (IEL) from a representative non-celiac patient (A) and a celiac patient (B) are shown. In both panels IEL were first selected according to CD45 and SSC (A1 and B1). From gated CD45+ cells, IEL T-cells were then defined by a combination of CD3 and CD103 (A2 and B2). This double CD3/CD103 positive population was further analysed for TcR with a combination of monoclonal antibodies defining TcR αβ or γδ (A3 and B3). Results are percentages of IEL T lymphocytes.
compared to other etiologies of duodenal enteritis examined in other groups that were evaluated. Possibly gluten intake causes greater intestinal damage with a high production of ATG causing an increased production of FOXP3 lymphocytes. Following the withdrawal of the causative agent, a high level of FOXP3-expressing cells can still persist, which is probably the baseline of impaired Treg cells, which after gluten intake favour the emergence of new immune and inflammatory responses of CD (Villanacci et al., 2011).

We have not observed an increase of FOXP3 levels in high-risk individuals or those with predisposition to CD represented by the FDR group. The HLA risk genotyping for CD has no relation to the observed values of FOXP3. In our study, celiac patients had high levels of FOXP3 lymphocytes, although FDR presenting genetic predisposition for the disease had levels of this molecule similar to those observed in the control group or with gastrointestinal functional pathology lacking this genetic component. The FOXP3 gene is encoded in the human genome although no relation to HLA DQ2 or DQ8 haplotypes associated to CD was observed (Hedrick et al., 2012).

Previous research has raised the possibility that *Helicobacter pylori* infection causes an increase in FOXP3 lymphocytes as an evasion mechanism of the immune response (Jang, 2010). Our data shows that the presence of *Helicobacter pylori* cannot act as a confounding factor since the presence of this bacteria is higher in FDR group and subjects with functional dyspepsia compared to the celiac group, therefore FOXP3 levels observed in our series does not seem to be caused by these bacteria.

Immunohistochemical analysis of FOXP3 and quantification lymphocytes T-γδ by flow cytometry can identify individuals with an increase of IELs, secondary to a gluten sensitive enteropathy. The increase in lymphocytes T-γδ by flow-cytometry analysis of fresh duodenal samples raises suspicion of CD in lymphocytic enteritis (Calleja et al., 2011). However, this procedure is complex, it consumes a large amount of economic resources and time, and is not available in most hospitals. Moreover, when both techniques are compared, we observed a correlation to diagnose CD in lymphocytic duodenitis. Microscopy study of immunohistochemistry requires an expert pathologist of CD, but can be performed in most health centres.

The main limitation of this study is the sample group size that affects the external validity of the results. However, by comparing the results obtained in celiac subjects with other groups such as the FDR, individuals with functional dyspepsia or most importantly the control group of asymptomatic subjects, it highlights the increase in consistency of our findings. In addition, it might be interesting to know how different factors are involved in FOXP3 expression in duodenal mucosa. On the basis of this data, the consumption of GFD or the eradication of *Helicobacter pylori* infection could be assessed after checking the number of FOXP3 lymphocytes before and after intervention.

Lymphocytic enteritis associated to celiac disease in the absence of villous atrophy presents an increased FOXP3 expression and gamma-delta lymphocytes, which are not detected in other etiologies of increased IELs. The determination of FOXP3 by immunohistochemistry is an easier and more feasible method than flow cytometry. It is necessary to perform further prospective and well-designed studies to validate this procedure and make modifications in the gluten-free diet.

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Conflict-of-interest statement. All authors declare no conflicts of interest.

References


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